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We have been studying animal/plant symbiosis using a model system of a single cell animal, the amoeba Trichosphaerium, and a unicellular plant, the symbiotic dinoflagellate Symbiodinium.

We have cultured the two partners as a consortium as well as independently. We have been describing the life history of the newly isolated and poorly known amoeba, and investigating its interactions with several species of these symbiotic dinoflagellates.

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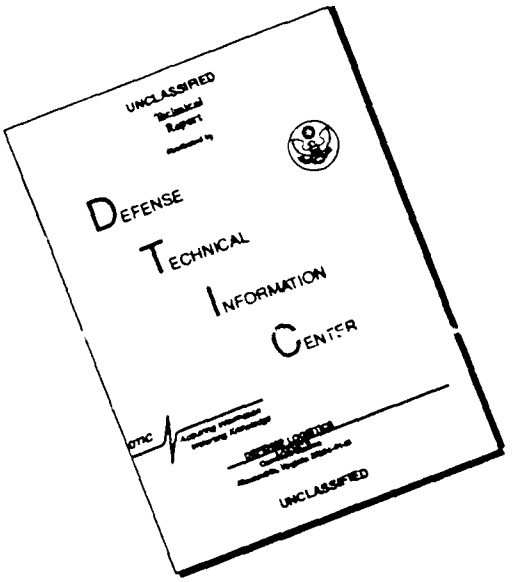

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Our major conclusions to date are:

1. The amoebal life history is composed of four distinct morphological forms the prominant one is a multinucleated form ranging in size from about 30-300 microns. This main morphological stage (Morph-I) is capable of creating the consortium with the Symbiodinium.

2. The amoebae recognize and differentiate four groups of algae in the genus Symbiodinium. One group of Symbiodinium species were digested by the amoeba with no ill effects to the animal cell. Another Symbiodinium species was toxic when phagocytosed. A third group was barely phagocytosed and mostly avoided, and the fourth group created symbiosis with amoebae which stuffed themselves full of these algae.

3. The amoebae do not only recognize the different algae but also replace less desirable algae already in their cytoplasm with more desirable (digestible and not toxic) species within 15 hours.

4. Two cytoplasmic vacuoles were distinguishible by differential lectin staining in the amoeba. Food vacuoles containing bacteria bound a variety of lectins while the membranes of perialgal vacuoles containing undigested symbiotic algae did not.

5. Like a true symbiont, the algae transferred photosynthetic metabolites to the amoebae and divided inside the cytoplasm.

6. Low maintenance - Long term cultures of the consortium have existed in culture as a closed system for over four years.

7. The selective digestion by the amoeba enabled the establishment of axenic cultures of the nondigestible algae. Contaminating microorganisms were eliminated by being digested while the intact non-dcgestible algal cells florished and were subsequently isolated and cultured axenically.

8. Enzymatic digestion of the algal surfaces by proteolytic enzymes, or coating them with lectins, darmatically changed the uptake rates and, in some cases, the speed of digestion indicating involvement of cell wall surfaces in the initial recognition stage and consortium formation.

June 17, 1991

A FINAL REPORT FOR THE PROJECT "SYMBIOTIC INTERACTIONS BETWEEN THE MARINE AMOEBA TRICHOSPHAERIUM AM-I-7 AND SPECIES OF THE SYMBIOTIC DINOFLAGELLATE SYMBIODINIUM".

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ABSTRACT

The large and multinucleated marine amoeba Trichosphaerium-I-7, and some species of the dinoflagellate Symbiodinium were used as a model system for animal/plant symbiotic interactions. These two unicllular partners which can be independently cultured, existed as a no-maintenance closed system for over four years. The amoebae actively phagocytosed the algae which were packed intact in amoebal cytoplasmic vacuoles within 1-5 days after infection. The amoebae received photosynthetic metabolites from the algae which helped maintain the animal cells at low division rates. Both partners were capable of being cultured independently when isolated from the consortium. The amoebal cytoplasm was packed with the algal cells some in groups of as many as 5 intact cells but mostly in single algal compartments. Dividing algal cells were observed in TEM sections, as were few dead cells. The amoebae were capable of digesting broken algal cells and wall preparations, as well as killed or coated algae. The recognition and uptake of the algae by the amoebae were grossely effected by enzymatic digestion and lectin treatments of the algal surfaces. The chemical nature of the changes on the algal surfaces which effect uptake is yet to be determined. This unique symbiotic interactions between the unicellular animals and unicellular plant cells is being persued for further investigations of the methods of communication between the interacting organisms.

We have been studying animal/plant symbiosis using a model system of a single cell animal, the amoeba Trichosphaerium, and a unicellular plant, the symbiotic dinoflagellate Symbiodinium.

Over the last three years we have cultured the two partners as a consortium as well as independently. We have been describing the life history of the newly isolated and poorly known amoeba, and investigating its interactions with several species of these symbiotic dinoflagellates.

Our major conclusions to date are:

1. The amoebal life history is composed of six distinct morphological forms all of which cycle around a multinucleated stage ranging in size from about 30 to over 200 microns. This main morphological stage (Morph-I, please see figures and diagram in manuscript Appendix I) is also the one capable of creating the consortium with the Symbiodinium (Polne-Fuller et al. Fig.17 Appendix I). Two of the transitions between the different forms depends on culture conditions and can be easily induced, these are the transitions between Morph-I and Morph-II and back to Morph-I; and between Morph-I and Morph-III and back to Morph-I. The others occure occasionally in cultures and the conditions for their inductions are under investigation.

Trichosphaerium spends most of its life in Morph-I as it grows from 20um up to over 200um in diameter. The cells feed actively and divide about once a day via binary fission. These originally colorless cells engulf Symbiodinium cells and become plump full and deep orange-brown. Such consortia maintained themselves for several years without media change.

2. The amoebae recognize and differentiate four groups of algae in the genus Symbiodinium. One group of Symbiodinium species were digested by the amoeba with no ill effects to the animal cell. They also digest well a large variety of non-symbiotic dinoflagellates such as Gonyaulax, Peridinium, and Gymnodinium. Another Symbiodinium species was toxic when phagocytosed. A third group was barely phagocytosed and mostly avoided, and the fourth group of algae created a consortium filling the cytoplasm of the animal cell. The ability of the amoebae to interact differently with the different algae which supposedly belong to one genus presents a system in which one can study the differences between these algae. Especially the roll of surface component(s) in the recognition phenomenon.

3. The amoebae do not only recognize the different algae but also replace less desirable algae in their cytoplasm with more desirable (digestible and not toxic) species within less that 15 hours. The exchange of the perialgal/food vacuole content occured gardually as amoebae full of symbiotic algae proceeded to engulf newly introduced digestible cells of Chlamydomonas or yeast, and simultaniously released the symbiotic dinoflagellates.

The released Symbiodinium was phagocytosed again three weeks later when the digestible algae were consumed and if no other digestible food was made available. In opposite experiments amoebae well fed on Chlamydomonas were exposed to Symbiodinium it became obvious that the exchange of the vacuoles content was controlled. Only minimal numbers of non-digestible algae were taken up by the fed amoebae who ignored the Symbiodinium as long as edible food source was made available (in preparation, paper #5).

4. Two cytoplasmic vacuoles were distinguishable by differential lectin staining in the amoeba. Food vacuoles containing bacteria bound a variety of lectins while the membranes of perialgal vacuoles containing undigested symbiotic algae did not. The possible existance of two different types of cytoplasmic vacuoels needs to be further characterized (Rogerson et al. Appendix II, in print).

5. Like a true symbiont, the algae transferred photosynthetic metabolites to the amoebae and divided inside the cytoplasm (Rogerson et al. 1989). The amoebae collected at least 12% of the fixed radioactive bicarbonate, the algae maintained about 60% of the lable in them and the rest 28% was lost. The missing radioactivity could have been lost to gaseous CO2 or to release content of food vacuoles as the cells divided (multiple fission) during the preparation of the consortia for counting (Rogerson et al. 1989 Appendix III).

6. The algae supported *low maintenance* - long term cultures, of the consortium which have existed in culture as a closed system for over four years. In such selfsuffecient amoeba+algae cultures the algae were present inside and out of the amoebae. Many algae in the cytoplam were in various division stages, and over 99% of the algae were intact and viable and culturable upon release (in perparation, paper #6).

7. As a result of the selective digestion the amoeba were useful for establishing axenic cultures of the nondigestible algal clones. Digestible contaminating microorganisms were eliminated while the intact algal cells were isolated and cultured axenically (Polne-Fuller 1991, Appendix IV, in print).

8. Enzymatic digestion of the algal surfaces, or coating them with lectins, daramatically changed the uptake rates and in some cases the speed of digestion indicating involvement of cell surfaces in the initial recognition stage of consortium formation (Polne-Fuller and Gibor, in preparation, paper #7).

9. Over 90% of the algae in the amoebal cytoplasms were intact as observed in light and electron microscopy. Over 98% of the algae developed colonies when released from the amoebal cytoplasm. The induction of multiple fission is a useful methods for separating between the amoebae and their symbionts, and results in viable populations of algae and amoebae. The morphology of the amoebae releasing their symbionts was transformed from Morph-I into the

uniform populations of 20um flat and fan-like Morph-II (Appendix I). Most of these small amoebae were not capable of taking up Symbiodinium cells and starved to death when edible food source was not made available. However, occasionally cells of Morph-II amoebae did engulf algae and survived on Symbiodinium alone creating consortia of one small amoeba containing 1-4 intact algal cells. The biology of the phenomenon of multiple fission and its relations to the amoebal ability to take up algae is under investigation (in preparation, paper #8).

Patents:

The nature of this project studying cellular recognition and symbiosis did not lend itself to patents so far.

During a meeting with the ONR patent lawyer earlier this year a request was made to check the possiblity for a patent filing for a uniqe "RECOGNITION" system. During a conversation with the University of Califronia patent officer Dr. Annie Yau-Young it was concluded that at this time we do not have an approperiate patent material. It was suggested that a disclosure may be possibly filed if future data indicates a unique nature of this system of recognition.

Publicatins:

1. Rogerson, A., M. Polne-Fuller, R. K. Trencn & A. Gibor 1989. A laboratory-induced association between the marine amoeba Trichosphaerium Am-I-7 and the dinoflagellate Symbiodinium #8. Symbiosis 27:229-241.

2. Rogerson, A., M. Polne-Fuller, & A. Gibor 1991. Lectin binding sites in marine amoebae. Archive. Fur Protistenk (in print).

3. Polne-Fuller, M. 1991. A novel technique for preparations of axenic cultures of Symbiodinium (pyrrophyta) through selective digestion by amoebae. J. Phycol. 27:324-337 (in print).

In preparations:

4. Polne-Fuller, M., L. Drake, J. Gibson, M. Katics & A. Gibor. Morphological forms in the life history of Trichosphaerium Am-I-7.

5. Polne-Fuller, M. & A. Gibor. Selective interactions between the marine amoeba Trichosphaerium Am-I-7 and symbiotic and non-symbiotic dinoflagelate and other unicellular algae.

6. Polne-Fuller, M. Low-maintenance/long-term closed-system cultures of the consortium Trichosphaerium-Symbiodinium.

7. Polne-Fuller, M., L. Haslehurst, R. K. Trenck & A. Gibor. Emzymatic manipulations of animal/plant recognition via cell-surface modifications.

8. Polne-Fuller, M., W. T. Eve, K. Kealy & A. Gibor. Methods of Multiple fission in the marine amoeba Trichospaherium Am-I-7.

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APPENDIX I

MORPHOLOGICAL FORMS IN THE LIFE HISTORY OF THE TESTATE AMOEBA *TRICHOSPHAERIUM* AM-I-7

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Key Words: Giant amoebae, marine amoeba, mini amoebae, morphology, multiple fission, *Trichosphaerium*.

ABSTRACT

Six major cell morphological forms occured in cloned cultures of the large marine amoeba Trichosphaerium Am-I-7. Morphology-I (Morph-I): The most "common" cells - about 40-250 microns in diameter, multinucleated, reproduced by binary fission. Morphology-II: Fan-like cells - 15-20 microns in diameter, 2-5 nuclei per cell, resulted from multiple fission of Morph-I cells. Morphology-III: Giants - 300-2000 microns, multinucleated, resulted from fusion of cells of Morph-I. Morphology-IV: Minies - about 5 microns long, uninucleated, resulted from extensive multiple fission of Morph-I cells. Morphology-V: Minigiants - 15-40 microns, complex net-like morphology resulted from fussion of mini cells. Morphology-VI: Clusters - 30-40 microns round cells originated from Morph-I, and usually attached to each other forming "grape-like" clusteres. The different morphologies appeared to be stages in the life history of this testate amoeba. The two small forms (Morph-II and IV) existed in sinchronous populations which had uniform number of nuclei and cell size. The other four morphologies were less uniform in size and number of nuclei, yet had distinct morphological characteristics. No sexual interactions (gamon stage) were confirmed, although interacting pairs of Morph-I cells, and mixing of cytoplasms were observed.

INTRODUCTION

The large multinucleated marine amoeba Trichosphaerium Am-I7 is a testate rhizopod that feeds on seaweeds (Polne-Fuller 1985). The genus is wide spread geographically and has been isolated from sand samples, debris, and seaweeds, from southern Brazil, northern and southern Australia, Hawaii, and California, by the authors, and from eastern USA (Spoon 1988) and Europe (Rogerson et al. 1989). Due to the limite published information availble on this genus, and the lack of studies in pure cultures, its life history remains partially unknown.

Trichosphaerium Am-I-7 has been cultured continuously in our laboratory since its isolation in the summer of 1985 (Polne-Fuller 1987). Over the years the species was re-isolated from local beaches, and several independent cell lines have been cloned and cultured.

The information presented here accumulated through culture work of these, cloned and cleaned, isolates under various culture conditions. We report of previously unknown morphological forms of this organism and their place in its life history. We also raise further questions which require further experimentation before a complete life history can be presented.

MATERIAL AND METHODS

The large, multinucleated marine amoeba Trichosphaerium Am-I-7 was isolated from live tissues of the brown seaweed Sargassum muticum (Polne-Fuller 1985). Single cells were isolated, cloned, and cultured on yeast, unicellular algae, or seaweeds, in sterile seawater (Polne-Fuller 1985).

Experimentation and observations of the different morphologies were made in flat plastic tissue-culture flasks or plastic perti dishes on an inverted microscope. For high resolution observations samples were transferred onto glass or depression slides and sealed with silicon grease. Four periodic feeding patterns were established. 1. Cultures were fed once a week on autoclaved unicellular algae such as Chlamydomonas or Gonyaulax. 2. Cultures were fed twice a month on autoclaved Bruer yeast. 3. Cultures were fed once every two months on kelp or other seaweeds. 4. Cultures did not need external feeding when co-cultured with live marine unicellular algae such as Chlorella, Dunaliella, or symbiotic dinoflagellates. The cultures were observed on a daily or weekly basis as required.

Observations of the different morphologies were described and recorded photographically during routine maintenance of the cultures. Specific experiments were performed to confirm these observations and to induce specific morphological stages.

Morphology-I - "Common" 2p cells (Fig. 1-3):

Cells of Morph-I were maintained as the common starting cultures for all experiments. They reproduced via binary fission (2P = 2 progeny) and were harvested as the food source was nearly depleted and the amoebae attached to the bottom of the culture

vessel.

Synchronized Morphology-II - Xp cells (Fig. 4-6):

To induce multiple fission, actively growing cultures of Morph-I were rinsed well in clean seawater. Observations before rinsing assured that the amoebae were well attached to the bottom of the culture vessle. If When too many cells were loose, vigorous shaking of the culture, followed by a 10 minutes of stationary settling, induced attachment. When large food particles loaded with amoebae were present, the particles were allowed to sink to the bottom of the culture flask and the top liquid carefully discarded. Such repeated rinsing diluted the medium sufficently to induce multiple fission as described below. Recently fed cells, with full food vacuoles, had to be rinsed about every 2-3 hours as they released the content of their food vacuoles. Additional rinses followed as needed to remove the freshly released, partially digested, food.

Morphology-III - Giants (Fig 7-8):

Giant amoebae formed when high densities of Morph-I cells were present. To induce giant formation, large numbers of Morph-I cells were placed in filter sterilized, "used" medium from well established amoeba cultures. This medium was saturated with water soluble food extracts and did not induce multiple fission. The dense cell populations were measured daily and their changing morphologies recorded.

Morphology-IV - Minies (Fig. 9-11):

Minies were observed occasionally in Morph-I cultures. Singl cell isolation of minies was facilitated by finely pulled glass capillaries. Isolated single mini cells, and groups of these cells were cultured in 8-well microscopic slides (Falcon...) as well as in tissue cultures flasks. The cells were fed either yeast or ground Macrocystis. The cultures were observed daily for two weeks.

Morphology-V - Minigiants (Fig. 12):

Minigiants were observed in dense cultures of Morph-IV mini-cells. These were observed microscopically and documented photographically.

Morphology-VI - Clusters (Fig. 13) :

Clusters of cells were observed occasionally in "old" and possibly starved Morphology-I cultures.

Preparation of food sources:

Brewer's yeast:

Dry Brewer's yeast was autocalved in screw-cap, glass test tubes. For routine culture, the autoclaved, caked yeast was dissociated into loose powder and about 100mg was disturbuted into the culture flasks of 50 cm^2 attachment surface. In quantitative feeding experiments, 100mg of autoclaved yeast powder was ground in 1 ml of sea water. The suspension was brought up to 100 ml sea water, and uniform amounts of suspension were added to the cultures (1 ml per 10ml culture medium).

Seaweeds:

Seaweeds were either dried and ground into fine powder in a mill (Polne-Fuller 1985, Polne-Fuller et al. 1989) or blended in an Hamilton blender, with Nanopure fresh water. Maceration of the tissues to about 200 micron pieces or smaller was carried out in a fine homogenizer. The powder, or the wet macerated tissues were autoclaved five times in fresh Nanopure water, to remove water soluble matter. The washed seaweeds were autoclaved two more times in seawater and divided into 20 ml screw cap glass test tubes. Each tube was 2/3 filled with seaweeds and sea water (1:1) and autoclaved (20 minutes, 120^{o}C, 250 psi).

Nuclear staining:

For staining the nuclei, cells were allowed to attach and strech on a flat surface for about 15 minutes. The attached cells were fixed with 1:3 acetic acid/alcohol. DAPI or Hochest (refs...) dissolved in Nanopure water were added (1ug/ml) and the amoebae were observed under a fluorescent microscope. Nuclear stains penetrated fixed cells easily and brightly stained the nuclei within less than 5 minutes. Quick vital staining with Hochest was performed in sea water. This dye was allowed to penetrate stretched live cells for 15 minutes before observations.

Low concentrations of DAPI, Hochest, and Acridine Orange, were also used successfully as vital dyes. Hochest was used at 10mg/ml, DAPI at 0.1mg/ml, and acridine orange at 0.01mg/ml. All three stains were added to the cultures one week before the experiments.

Time-lapse photography:

Motion of amoebae and their feeding habits were observed through a time-lapes photographic system. A Mitchubishi Time-lapse recorder and a Mitchubishi color movie camera were used. Exposures were taken three times a minute and observed at real time.

RESULTS AND DISCUSSION

Observations over five years of culture and a wide range of experimentations revealed six distinguishable morphologies of Trichosphaerium Am-I-7. These morphologies were related to the growth and the life history stages of the cultured amoebae.

The order in which the following morphologies were presented is somewhat arbitrary. The selection of "Morphology-I" as the first to be numbered was due to its being the dominant cell form in growing cultures and the most common morphology from which other formes differentiated.

Morphology-I - 2p cells:

These cells divided by binary fission, each mother cell producing two similar progeny which were not always identical in size. Morph-I was previously documented and published as the normal Trichosphaerium morphology (Angell 1976) which were usually found in field samples.

Cell sizes of this morphological form ranged from 30 to over 250 microns, and contained 10 to over 270 nuclei, where larger cells contained a larger number of nuclei. Attached cells became flater and larger as they stretched on the substrate. Wide lobopodia were actively extended constantly changing the morphology of the amoeboid cell. Filipodia were forming, slowly appearing and disappearing, and their numbers increased in the direction of motion. Detached cells became round and their filipodia were extended into the surrounding medium in all directions, thereby, holding the cells above the substrate. Movement of detached cells was performed by shifting cell weight

from filipodia which were in contact with the substrate to newly extended ones. This type of motility lasted about 10-15 minutes until the cells re-attached and flattened. A few cells attached as soon as 2 minites after stationary conditions, while the majority of the cells required about 15 minutes to establish stable attachment.

Active cytoplasmic streaming was observed on time-lapse recordings. Cytoplasmic activity was a good indication of cell viability at all morphological stages. Another useful indication of viability was the close contact of the test to the cytoplasm. Swelling of the tests was always an indication of ill health, although at times a rinse with clean sea water reversed the condition and saved the cells from death.

On two isolated occasions pairs of Morph-I cells were observed in otherwise regular cultures (Fig. 16). The pairs remained in contact for at least nine hours, and disappeared by the next day. The function of this distinct and uncommon pairing formation was not determined, although interactions between cells in less distinct formations was common.

Another uncommon morphology which was found occasionally in Morph-I cultures was amoebae containing smaller, intact, amoebae inside their cytoplasmic vacuoles. The smaller amoebae were alive and their filipodia extended and active. The interaction between the large containing cell and the smaller contained amoebae was either an uncommon vegetative propagation, possibly leading to morphology IV (see below), or a case of larger cells preying on smaller cells. Preying-like activities were observed on several occasions in both naked Trichosphaerium and spicuoled species.

Morphology-II - Xp cells (Fig. 4-6):

Cells of Morph-II were termed XP cells expressing the variable number (X) of Progeny which they produced through their multiple fission.

Induction of multiple fission was reliable and simple to perform, although it biochmical process is not understood. The phenomenon can be reliablly induced by rinsing Morph-I cells with clean sea water. It is not clear whether the induction of this multiple fission involved a removal of an inhibitory factor(s) or an introduction of an inducer(s). Extensive dilution of "used" medium and its replacement with sea water was the most reliable way for inducing XP, however, at times stresses of other kined also resulted in multiple fission. An examples was a two hour gentle but constant shaking of cultures during travel, which resulted in an XP population. In contrast, similar shaking on a rotary shaker at room temperature in the laboratory did not induce multiple fission. On several occasions, starved cultures also contained as many as 50% XP cells however, their neighboring cells remained in Morph-I.

Multiple fission was inhibited by yeast, seaweed cell extracts, the content of amoebae food vacuoles, as well as by highly concentrated sugar solutions.

Cells of this morphology were uniform in size (20 microns), fan-like, with a denser cytoplasm area ocuping half of the cell and a clear fan-like lobopodium extended at the direction of motion. Filipodia were not common in these small cells, although they were seen extended from the lobopodium occasionally.

Detached Xp cells became round and extended filipodia to the medium as did the Morph-I cells.

Xp cells contained 1-3 nuclei (rarely one) all present in the dense cytoplasmic area. The cells started feeding 1-3 days after their division and developed into Morph-I cells within one week. Single isolated XP cells were cultured to determine whether each can develop independently into Morph-I. All 50 isolates developed into normal Morph-I amoebae indocating that these are not a sexual gamont stage.

Morphology-III - Giants:

Giant amoebae formed when large densities of Morph-I cells were present. They reached sizes of as large as 3000 microns and their nuclear numbers were too large to count (over 1000). Giants existed in stretched morphologies as well as large solid patches (Figs. 7,8). Many were pale indicating inactive feeding. To induce giant formation, large numbers of Morph-I cells were concentrated in culture flasks or petri dishes. This procedure had to be performed in "used" medium, saturated with water soluble food extracts, to avoid induction of multiple fission.

Patches of giant amoebae were formed within 2-4 days after transfer to dense cultures. The giants were formed by aggragations of Morph-I "common "amoebae which fused their tests and cell membranes. Mixing of two live populations of amoebae, one with nuclei stained by DAPI and the other by light acridine orange, indicated that the nuclei of giant cells streamed into neighboring cytoplasm through the first narrow bridges which were

formed. This exchange of cytoplasm occured even before the tests and cell membranes completed their fusion. Cytoplasmic exchanges were commonly seen among the many cells forming the giants, however, single Morph-I cells which were in contact with only one other cell of the same morphology, were also likely to exchange cytoplasm in densely populated cultures.

Giants lasted for about one week and then seperated back into Morph-I cells. Similar interactions of bridge formation, mixing of cytoplasms, and re-separation occured in dense population of Morph-I cells even if giants were not formed.

Morphology-IV - Minies:

Minies were the smallest size cells which were observed in the life history of Trichosphaerium-I-7. They were about 5 micron long (Fig. 10), uninucleated, and emmerged in large numbers from the tests of morphology I cells (Fig 11). Induction of minies in the laboratory was not reliable although they appeared in well fed cultures and more frequently in starved ones. The cytoplasm of minies was smooth and uniform, and did not produce lobopodia or filipodia. Their common pattern of motion resembled that of a large spiral bacterium with occasional amoeboid movement as the cell changed its direction of travel.

Twenty five samll groups of minies (5-12 amoebae in each group) were isolated and cultured. All of these grew back into "common" amoebae of morphology I. However, only two of twenty five single isolated minies developed into morphology I amoebae while the other twenty three lived for about a week, did not feed, and died. The chance of having two mini amoebae tranferred

together or two cells which fused before they were transferred is not impossible. Therefore, it is not clear whether isolated, single minies of Trichosphaerium are capable of growing into morphology I amoebae, or whether they may be a gamont form requiring a mate.

Morphology-V - Minigiants:

Minigiant amoebae were observed in dense cultures of morph-IV minies (Fig. 12). They were never intentionally induced nor where they observed to develop into morph-I amoebae. However, minigiants were observed at length by microscopy and time-lapse photography. Their migration out of the mother cell's test lasted about 24 hours, and their agregation into minigiants, an active migration and fusion, occured with in 3-5 days after mini formation. Minis existed in a variety of sizes, from single cells (3-5 micron long) to 2 or more fused cells (7-15 micron), and up to the minigiants, a reticulate form of up to 40 microns in diameter. Since small groups of minies regenerated into morph-I. Since minigiants were also aggregates of mini cells, it is likely that minigiants may also be able to grow into the "common" morph-I form.

Morphology VI - Clusters:

Clusters were observed occasionally in older, starved, morph-I cultures with high concentration of degraded food particles (Fig. 13-14). They appeared as groups of about 40 micron amoebae clustered together attaching to each other. A few of the amoebae at the bottom of the clusters were attached to the

substrate. Clustered amoebae spent days, possibly weeks at this morphology before they returned to the attached morphology I stage and started feeding again.

CONCLUSIONS

Trichosphaerium I-7 survived perpetual subculturing of its Morph-I form for the last five years. However, at least five other morphologies did exist in its life history and four of them are known to re-differentiate into the common morph-I cell type. Fig. 17 summarizes the source of differntiation and the target form for each of these morphologies. It is interesting to note that four of these morphologies returned to the source form of Morph-I, there being Morph-I,II,III, and VI. Morph-IV did returned to Morph-I when more than one mini cell was present, and Morph-V, although was not observed returning to Morph-I would be likely to do so, since it in itself formed from fusing Morph-IV cells.

Several questions still remain unanswered. We do not know the exact sequence and function of the different morphologies of the life history of this protozooan in the field. We do not know whether any of these forms are necessary for the long term survival of the species, or whether any of the morphologies interact as sexual entities. It is also unknown whether the nuclei in the mixing cytoplasms were fusing. Ecologically it would make sense that both forms of multiple fission, and the clusters are advantageous for dispersle of a large number of germplasm at time of stress The ecological advantage of giants is not clear although their function as a mixing pot for nuclei

of many cells may have some genetic advantages.

During the present study all of the amoebae were of naked, non-spicuoled morphology. We have not observed the appearance of spicuoled forms in the cultures of any of these morphologies at any time and under any culture conditicns. This suggests that the known spicuoled form of Trichosphaerium (Sheeham & Banner 1973) is probably a differnet species than the naked form with which we are working, and may not be an agamont. However, in cultures of spicuoled isolates we did observe appearance and disappearance of partially spicuoled, sparcely spicuoled and seemingly naked amoebae. Further work on the spicuoled species is in progress.

Acknowledgements

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REFERENCES

Angell, R.W. 1975. Structure of Trichosphaerium micrum sp. J. Protozool, 22:18-22.

_____. 1976. Obsrevations on Trichosphaerium platyxyrum. J. Protozool. J. Protozool. 23:357-364.

Olive 1970. The Mycetozoa: a review classification. Bot. Rev, 36.

Olive LS. & Stoianovitch C. 1960. Two new members of the Acrasiales. Bull. Torrey Bot Club 87: 1-20.

Polne-Fuller, M. 1987. A multinucleated marine amoeba which digests seaweeds. J. Protozool. 34:159-165.

Rogerson, A., M. Polne-Fuller, R.K. Trench, and A. Gibor, 1989. Symbiosis 7:229-241.

Schaeffer AA. 1926. Taxonomy of the amoebas. Carnegie Inst. Wash.Publ. 345, 1-116.

Spoon, 1980 abstract J. protozool.

Table I: MORPHOLOGICAL CHARACTERISTICS OF THE DIFFERENT FORMS

	size/um	div.	nuc.	period	fili.	lobo.
Morph-I	30->200	2p	5->200	cont. months/ years (?)	0->10	0->5
Morph-II	15-20	Xp	3-5	3-7days	rare	1
Morph-III	>300-5000	Xp	>300	4-9days	many	few
Morph-IV	2-6	none	1	5-10days	none	none
Morph-V	10-30	?	>20	7-14days	none	none
Morph-VI	40-60	none	20-50	7-30days	many	rare
Pairs	60-80	none	40-70	1-5days	few	none

	feeding	food vac.	motion
Morph-I	yes	yes	7-12um/min
Morph-II	yes	none	10-20um/min
Morph-III	no	yes	cytoplasmic
Morph-IV	?	none	30-40um/min
Morph-V	yes	none	?
Morph-VI	no	few	cytoplasmic and local
Pairs	no	yes	cytoplasmic

FIGURE LEGENDS:

Fig. 1. Cells of the commonly present Morphology-I (Morph-I) of Thrichosphaerium. Lobopodia (clear arrows) and food vacuoles were commonly present. Occasional large vacuole(s) accumulated "feces" (dark arrowheads). Dactylopodia were also common. Bar = 40 um.

Fig. 2. A dettached cell of Trichosphaerium which was fed agar on which Symbiodinium (a dinoflagellate) was cultured. Detached cells extended many filipodia (dark arrows) which protruded through a "volcano-like" cone (clear arrows). The granularity of the cytoplasm is due to the agar packed in many food vacuoles. Several cells of the alga Symbiodinium were also phagocytosed (dark arrow heads). Bar = 15 um.

Fig. 3. Binary fission in Trichosphaerium fed on Chlamydomonas. The narrow cytoplasm (dark arrow) evantually became thread thin before disconnecting (see Fig. 6). Released and partially digested algal cells can be seen at the bottom left (clear arrow). A progeny of another binary fission is present at the top right (black arrow head), its brother progeny is just above him and can be partially observed at the top right edge of the photo. Bar = 10 um.

Fig. 4. Progeny of one amoeba which completed multiple fission Morph-II. Rinkled cells without a lobopodium are the immediate product of the last fission (dark arrow heads). Within 3-10 hours each cell stretched and become motile extending a lobopodium (dark arrows). Dactylopodia with very

small or invisible cones were sometimes present. Bar = 25 um.

Fig. 5. A close up of stretched motile progeny of multiple fission. Twenty four hours after fission the cells were motile and polarized. A clear lobopod attached to the substrate via cytoplasmic pads in the direction of motion (clear arrows), and dense cytoplasm containing organels was ready to roll off the substrate (dark arrows) in the process of motion. Bar = 10 um.

Fig. 6. Stretched amoebae during the first 3-7 hours of multiple fission. The cells released their food content and have been dividing repeatedly. Thin cytoplasmic strings (dark arrows) about to break apart, are the last bridge between the dividing cytoplasm. A small nonmotile cell (clear arrow), typical progeny of multiple fission is marked by the celar arrow. Dactylopodia can also be seen. Bar = 10 um.

Fig. 7. Two giant Trichosphaerium (Morph-III, black arrows) surrounded by Morph-I cells (clear arrows). The giants are lighter in color, an indication of lack of food in the cytoplasm. The smaller is 200x400 um, the larger 800x1,400 um. The size of the surrounding cells range between 40-150 um. The giants were formed by fusion of Morph-I cells. Bar = 150 um.

Fig. 8. A giant in the process of formation. Present length is 2,560 um. The cell test and membrane are distinct as the cell releases the content of its food vacuoles (clear arrow). The reticulate nature of the merging cells may be

elaborate. Giants as large as 5 mm in diameter were observed. Bar = 40 um.

Fig. 9. Mini cells (Morph-IV, 2x3-5 um, dark arrows) immerged out of a Trichosphaerium similar to the one in the center of the field (clear arrow). Their snake-like motility isa expressed in their generally sigmoid shapes. Bar = 15 um.

Fig. 10. Mini cells three days after release. What were uniform, 2x5 um cell at release (larger clear arrow), have fused into cells of variable sizes. Fusents of two and more mini cells can clearly be distinguished (dark arrow heads). Some assume amoeboid forms and motion. A 20 um cell, a progeny of multiple fission (Morph-II), presents a good reference for size and form (dark arrow). Bar = 6 um.

Fig. 11. Transformed Morph-I cells (clear arrows) releasing mini cells (dark arrows). Bar = 10 um.

Fig. 12. A mini-giant (Morph-V) formed a week after release of mini cells. It is not known whether the reticulate form was created by the fusing mini-cells or by secondary motility of the already formed giant. Bar = 15 um.

Fig. 13. Clustered amoebae (large clear arrow) attached to each other as well as to the substrate. Clusters formed in cultures where the cells were starved and the only particles in the vessel were amoebal feces (small clear arrow). Occasionally cells detache from the cluster and migrate away or settle within close vecinity to the source cluster (dark arrow). The later were likely to move back and reattach to the cluster.

Fig. 14. Dedifferentiating cluster. Two to four week old clusters did disperse and their cells became motile and migrated in search of food. Allatched cells formed lobopodia at the direction of motion (dark arrow). When cluster dedifferentiation occured, all of the cluster cells went through the process within no longer than 5 minutes. Time lapse photography revealed that the rounded cells (clear arrow) which formed the body of the clusters were healthy and their cytoplams in constant motion.

Fig. 15. Trichospaherium cells containing cyst-like round cells (dark arrow) were occasioanlly observed. These were alive, as indicated by their streaming cytoplasm. However, they were not seen to immerge out of the mother test (dark arrow heads). Bar = 10 um.

Fig. 16. Rare formation of pairs was observed on three different occasions. The paired cells remained paired for at least one week, and then parted. Their morphology and general motion after detaching from each other were undistinguishable from morph-I cells.

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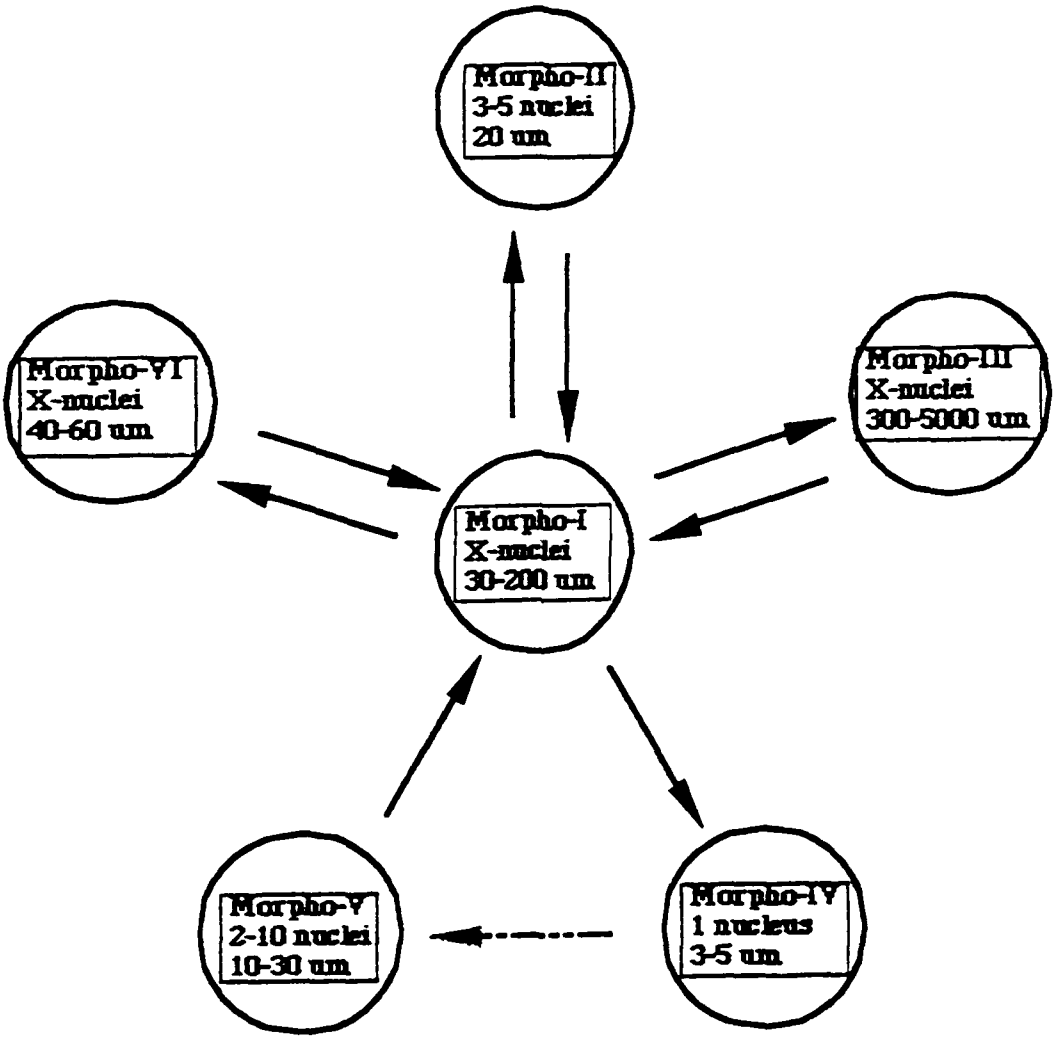
Morpho-II
3-5 nuclei
20 um
Morpho-VI
X-nuclei
40-60 um
Morpho-III
X-nuclei
300-5000 um
Morpho-I
X-nuclei
30-200 um
Morpho-V
2-10 nuclei
10-30 um
Morpho-IV
1 nucleus
3-5 um

Fig. 17

APPENDIX II

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Lectin Binding Sites in Marine Amoebae

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key words: lectins; marine amoebae; identification
With 14 Figures

Summary

The binding characteristics of 7 different FITC-conjugated lectins with 7 species and 1 strain of marine amoebae were examined. This survey identified 8 different binding compartments in the cell. Although there was little congruity in binding action, even within a single genus, the survey did highlight cases where lectin binding has diagnostic application in the identification of closely related and morphologically similar amoebae. Moreover, one strain of amoeba, *Paramoeba* HB showed unique cytoplasmic binding with *Triticum vulgaris* agglutinin (WGA). The fine structure of this amoeba was examined and a vesicular cytoplasmic region was identified as the probable site of WGA-binding. The applicability of lectin binding for the identification and study of marine amoebae is discussed.

Introduction

Lectins are proteins which bind specifically to carbohydrate moieties of complex carbohydrates, glycoproteins and glycolipids. First described from the seeds of plants, lectins are now known to be widely distributed among viruses, bacteria, plants and animals (GOLD and BALDING 1975) where they exhibit an array of interesting biological and chemical properties. However, it is their capacity to act as cell surface recognition molecules that has aroused most interest (e.g. WEIR 1980) and they are now popular biochemical tools in cell biology often finding application as discriminatory agents (ETZLER 1985).

Lectins have been used to identify sugar residues in a variety of free-living protozoa, particularly the ciliates *Paramecium* (MERKEL et al. 1981; LUTHE and PLATTNER 1986; LUTHE et al. 1986; ALLEN et al. 1988), *Euplotes* (LUEKEN et al. 1981), *Stentor* (MALONEY 1984, 1986, 1988) and *Tetrahymena* (FRISCH and LOYTER 1977; WATANABE et al. 1981). To date, no free-living marine amoebae have been examined for lectin-binding activity although Con A has been used to determine the involvement of glycoproteins on the cell surface of freshwater *Acanthamoeba* during phagocytosis (BROWN et al. 1975), to study surface compartments involved in the locomotion of *Naegleria* (PRESTON et al. 1975) and to distinguish between species of *Naegleria* (De JONCKHEERE et al. 1984).

We reasoned that the specificity of action of lectins could make them powerful biochemical tools in the identification of naked amoebae; a difficult group to identify to species because of the lack of objective diagnostic features (ROGERSON 1988). Thus, in an attempt to define diagnostic applications of lectin binding in amoebae, this descriptive study examines binding sites in

amoebae from different genera. The study follows and complements an earlier investigation of novel cytological staining methods which have application in the identification of marine gymnamoebae (ROGERSON 1988).

Materials and Methods

Cultures. Cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP), Windermere, England, United Kingdom. Detailed procedures on culture methods are given elsewhere (PAGE 1983) and only brief details are included here. *Mayorella kuwaitensis* (CCAP 1547/1) and *M. gemmifera* (CCAP 1457/8) were cultured in Føyn's Erdshreiber medium and C75S medium respectively with bacteria (an unidentified species of *Pseudomonas*), fungi and flagellates. *Paramoeba pemaquidensis* (CCAP 1560/4), *P. aestuarina* (CCAP 1560/7) and *P. invadens* (CCAP 1560/8) were cultured on MY75S agar with *Pseudomonas*; *P. eilhardi* (CCAP 1560/2) was grown in Føyn's Erdschreiber liquid medium with the same bacterium. Two additional amoebal isolates were examined. An undescribed species of *Paramoeba*, morphologically similar to *P. pemaquidensis,* was collected from below the sea ice in Hudson Bay, Canada by Dr. M. Lewis of Dalhousie University, Halifax, Nova Scotia, Canada. Clones were isolated and maintained on MY75S agar with *Pseudomonas*. This *Paramoeba* has been used in a previous study where it was designated research strain HB. *Trichosphaerium* AM-1-7 is a marine isolate from California, maintained in liquid culture (C75S with bacteria) and described by POLNE-FULLER (1987).

Lectin binding. Lectins conjugated with fluorescein isothiocyanate (FITC) were purchased from Vector Laboratories (Burlingame, CA, U.S.A.). These, together with their nominal sugar specificities (ARAVIO et al. 1980), were as

follows: *Ulex europeus* agglutinin (UEA-1;α-L-fucose), concanavalin A (Con-A; α-D-glucose and α-D-mannose), *Ricinus communis* agglutinin (RCA 120;β-D-galactose), *Dolichos biflorus* agglutinin (DBA; N-acetyl-α-D-galactosamine), *Triticum vulgaris* agglutinin (WGA; N-acetyl-β-D-glucosamine) and *Arachis hypogaea* agglutinin (PNA; N-acetyl-D-galactose-β-galactose).

To investigate lectin-carbohydrate interactions in amoebae, cells from exponentially growing cultures were washed off agar surfaces with sterile filtered seawater or decanted from liquid cultures and concentrated by gentle centrifugation. Cells were washed 2 times in seawater before fixing in 8% formaldehyde (diluted with filtered, sterile seawater) for 30 min. This fixation protocol ensured that membranes were poorly preserved thereby allowing penetration of lectins into the cell. After 3 washes in sterile medium, cells were concentrated in 1 ml of medium to which 40 µg of lectin was added. Amoebae were labelled for 30 min, washed 3 times in fresh medium and examined on a Zeiss Photomicroscope III with epifluorescence. Photographs were recorded on Kodak Tri-X film with exposure times between 10 and 90 s.

Electron Microscopy. The *Paramoeba* HB isolate showed a unique cytoplasmic staining feature with WGA. In an attempt to identify this cytoplasmic structure at the ultrastructural level, cells were examined by TEM. Amoebae were fixed simultaneously for 20 min in 2% glutaraldehyde and 1% osmium tetroxide made up in a 50:50 mix of seawater and 0.1M sodium cacodylate buffer (pH 7.2, temp. 4 C). After 4 washes in cold distilled water, cells were dehydrated through a graded acetone series (30% to 100%) and embedded overnight in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 TEM.

RESULTS

Lectin-binding identified a total of 8 different cellular compartments among the range of amoebae examined. These are listed in Table 1 and examples of each are illustrated in Figs 1 to 8. They include the cell surface, cytoplasm, digestive vacuoles (ie. membranes and/or contents), nucleus, nucleolus, nuclear membrane, parasome and cytoplasmic vesicular regions.

The most consistent lectin-carbohydrate binding pattern occurred between Con A and the cell surface. In amoebae this surface, or glycocalyx, is often structurally complex (Fig. 12). The uniform bright fluorescence (Fig. 1) clearly delineated the morphology of the cell and occasional surface features such as the pores (Fig. 2) of the walled amoeba *Trichosphaerium*. None of the other lectins surveyed gave the same degree of binding consistency as Con A; nevertheless, some generalizations can be drawn.

The genus *Paramoeba* was unique in that some component of the nucleus, usually the nuclear membrane, bound Con A (Table 1). However, it should be noted that while none of the other genera showed nuclear binding of Con A, the possibility of a weak interaction being masked by the intense wall binding, characteristic for this lectin, cannot be discounted.

The lectin UEA-1 labelled the cell surface, cytoplasm or nucleus of most of the *Paramoeba* spp. (Table 1). A notable exception to this trend was found in the case of *Paramoeba* HB which showed no binding with UEA-1. This negative result is a useful observation for the histochemical identification of this particular isolate. Similarly, the cell surface and nucleus of *Mayorella kuwaitensis* bound UEA-1 whereas no binding was evident in the morphologically similar species *M. gemmifera*.

The lectin RCA 120 bound to components of the digestive vacuole compartment of *M. kuwaitensis* and *Trichosphaerium* and to the cytoplasm and

nucleus of both *Mayorella* spp., but failed to bind to any of the *Paramoeba* spp.

The lectins SBA, DBA and PNA all showed variable binding action. Many of the amoebae examined, notably the mayorellas and *Trichosphaerium*, did not bind these lectins (Table 1). Where positive results were observed, surface and/or cytoplasmic interactions predominated. Again, it is the anomalous results that are of greatest interest. For example, the results in Table 1 show that the binding of PNA to the nucleus of *P. pemaquidensis* distinguishes it from the other paramoebas and that nuclear binding of DBA distinguishes *P. invadens* from the morphologically similar *Paramoeba* (HB) and *P. pemaquidensis*.

The lectin which showed the greatest range of binding action was WGA. In many instances, specific components of the nucleus were stained (Table 1). This lectin also bound, albeit unpredictably, to the cell surface, cytoplasm or digestive vacuoles of the different amoebae surveyed. In *P. eilhardi* and *Paramoeba* HB, WGA stained the parasome, the DNA-rich intracellular bodies characteristic of amoebae belonging to the genus *Paramoeba*. An intriguing, and unique, binding pattern with WGA was observed in Paramoeba HB. Two brightly fluorescent regions were a prominent feature in the majority of cells examined. These unique sites were located in the cytoplasm, frequently in pairs close to the nucleus and parasome (Fig. 8).

To further determine the nature of the WGA-binding sites and general ultrastructural features of *Paramoeba* HB, cells were examined by TEM. The cell surface of this amoeba is covered by a glycocalyx composed of tightly packed tubular elements (Fig. 12). Few hairs or filaments were detected on the surface. The spherical nucleus had a prominent central nucleolus. As is often the case in this genus, the nucleus was closely apposed to the parasome, partially surrounding it (Fig. 9), however, no connections between these structures were found. The parasome had two end poles (only one is shown in the oblique section, Fig. 9) and a middle region containing fibrillar material. The cytoplasm

was characterised by numerous vacuoles and vesicles (Fig. 10). Vacuoles recently phagocytosed, i.e. still possessing an intact glycocalyx, had an angular appearance (Fig. 11). Mitochondria were elongate, about 1 μm in length, with tubular cristae. No Golgi stacks were observed although a vesicular region with possible similar function was found in the cytoplasm adjacent to the nucleus (Figs. 13 - 14). The location, size (1.0 - 1.5 μm) and paired nature of these vesicular inclusions suggests that they are the sites of WGA-binding observed in this strain of *Paramoeba*.

DISCUSSION

Binding of Con A to the cell surface of amoebae was dramatic, but hardly surprising in view of the glycoprotein-rich cell coat (glycocalyx) which covers most amoebae (PAGE, 1983). Although the morphology of these coats is markedly different between genera (surface structure being one of the most objective diagnostic characters in the identification of amoebae) they share a common binding affinity for Con-A. This indicates some chemical conservation in the glycoprotein coat of dissimilar marine amoebae, at least in terms of the exposed α-D-mannose and α-D-glucose moieties. It should be remembered, however, that Con A is broader than other lectins in terms of specificity and it will also bind to closely related sugars (SHARON et al. 1979). Similar surface binding with Con A has been reported for the freshwater amoeboflagellate *Naegleria* (PRESTON et al. 1975). While uniform surface binding of Con A is not unique to amoebae, most studies with other protozoa report less regular binding of this lectin. Typically, binding sites are either less distinct or restricted to specific regions of the cell surface (FRISCH and LOYTER 1977; LUEKEN et al. 1981; WATANABE et al. 1981; ALLEN et al. 1988). The delineation of the entire surface and/or cytoplasm of amoebae with FITC-Con A, regardless of

species, may have application in the enumeration of amoebae from open water samples and from experimental cultures. If the levels of organic floc and debris are low in the samples, it should be possible to discern fluorescing amoebae in fixed and stained material in the same way that the fluorochrome DAPI has been used for visualising nano- and pico-plankton collected on filters (eg. FINLAY et al. 1988).

In the most recent classification scheme proposed for the naked amoebae (PAGE 1987), *P. pemaquidensis* and *P. aestuarina* have been transferred to the new genus *Neoparamoeba*. This leaves *P. eilhardi* in the family Paramoebidae being more closely related to the genera *Mayorella* and *Dactylamoeba*. Although the present study was not designed to examine taxonomic relationships, it is worth noting that on the basis of lectin binding, *P. eilhardi* is more similar to the paramoebas than to *Mayorella* (Table 1, Con A and RCA 120). Further work with lectin binding may help in these distinctions but for the present we have retained the genus name *Paramoeba* for all those species containing a parasome.

In all *Paramoeba* surveyed in this study Con A bound to some component of the nucleus, usually the nuclear membrane. Similar findings have been reported for *Paramecium* (ALLEN et al. 1988) and in vertebrate cells where glycoproteins with Con A binding affinity are major components of the nuclear membrane pore complex (GERACE et al. 1982). However, the lectin with the greatest affinity for nuclear components in amoebae was WGA, an acetyl glucosamine specific lectin. Here, the nuclear membrane and nucleolus were common binding sites which is consistent with binding in mammalian cells. Glycoproteins of the nuclear pore complex bear N-acetyl glucosamine groups (TORRES and HART 1984) as do some glycoproteins of the nuclear interior, nuclear envelope and cytoplasm (HOLT and HART 1986).

Most of the other lectins examined failed to exhibit the same degree of consistency of action that was seen for Con A and for WGA. Nevertheless, these

lectins, if treated on an individual basis, are interesting since they give binding patterns that have application in selected research areas. For example, the ability of RCA 120 to delineate digestive vacuoles in *Trichosphaerium* has allowed the histochemical separation of digestive vacuoles from perialgal vacuoles containing endosymbiotic dinoflagellates in this amoeba (ROGERSON et al. in press). The lectin survey also provides novel diagnostic features for the rapid identification of morphologically similar amoebae. For example, the binding of UEA-1, a fucose-binding lectin, to the surface of *M. kuwaitensis* but not to the cuticle of *M. gemmifera* was unexpected, given the ultrastructural similarity of these two surface coats, but fortuitous since it provides a method for distinguishing between these alike mayorellids. Similarly, UEA-1 and DBA failed to bind to *Paramoeba* HB yet labelled *P. aestuarina, P. invadens* and *P. pemaquidensis* thereby providing a rapid method for distinguishing this isolate from the other common species. Moreover, while the binding of Con A to the parasomes in only three out of the five paramoebas is diagnostically interesting it also suggests that these endosymbiotic inclusions have different chemical fingerprints and thus may be of polyphyletic origin.

Although *Paramoeba* HB resembles *P. pemaquidensis*, lectin-binding has shown several important differences which may warrant a new species description for this isolate in the future. In particular, WGA identified two bright fluorescent regions in the cytoplasm of the majority of cells examined. At the ultrastructural level, two cytoplasmic vesicular regions were found which, because of their size and location, were the probable sites of WGA-binding. It is possible that these bodies function as Golgi complexes perhaps glycosylating the WGA-binding glycoproteins. One of the unique features of *Paramoeba* HB is that it was isolated from below the sea ice where temperatures are typically less than 0°C. It is tempting to speculate, therefore, that these vesicles contain localisations of glycoprotein cryoprotectants, similar to the glycoproteins found in the sera of most Antarctic fishes (DeVRIES 1971).

While the present study did not show a high degree of conservation of lectin-binding across the genera and species examined it did highlight some interesting lectin interactions which could be used for distinguishing between alike species and as a biochemical tool in selected studies requiring the histochemical localisation of cellular formed elements.

Acknowledgements

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Literature

ALLEN, R.D., UENO, M.S., and FOX, A.K. (1988): A survey of lectin binding in *Paramecium.* J. Protozool. **35:** 400-407.

ARAVJO, F.G., HANDMAN, E., and REMINGTON, J.S. (1980): Binding of lectins to the cell surface of *Trypanosoma cruzi.* J. Protozool. **27:** 397-400.

BROWN, R.C., BASS, H., and COOMBS, J.P. (1975): Carbohydrate binding proteins involved in phagocytosis by *Acanthamoeba.* Nature **254:** 434-435.

De JONCKHEERE, J.F., PERNIN, P., SCAGLIA, M, and MICHEL, R. (1984). A comparative study of 14 strains of *Naegleria australiensis* demonstrates the existence of a highly virulent subspecies: *N. australiensis italica* n. spp. J. Protozool. **31:** 324-331.

DeVRIES, A.L. (1971): Glycoproteins as biological antifreeze agents in Antarctic fishes. Science **172:** 1152-1155.

ETZLER, M.E. (1985): Plant lectins: molecular and biological aspects. Ann. Rev. Plant Physiol. **36** : 209-234.

FINLAY, B.J., BERNINGER, U-G., CLARKE, K.J., COWLING, A.J., HINDLE, R.M. and ROGERSON, A. (1988): On the abundance and distribution of protozoa and their food in a productive freshwater pond. Europ. J. Protistol. **23:** 205-217.

FRISCH, A., and LOYTER, A. (1977): Inhibition of conjugation in *Tetrahymena pyriformis* by Con A. Localization of Con A - binding sites. Exp. Cell Res. **110:** 337-346.

GERACE, L., OTTAVIANO, Y., and KONDOR-KOCH, C. (1982). Identification of a major polypeptide of the nuclear pore complex. J. Cell Biol. **95:** 826-837.

GOLD, E.R., and BALDING, P. (1975): Receptor-specific proteins. Plant and animal Lectins. Excerpta Medica, Amsterdam.

HOLT, G.D., and HART, G.W. (1986): The subcellular distribution of N-acetylglucosamine residues on the surface of intact lymphocytes. J. Biol. Chem. **261:** 8049-8057.

LUEKEN, W.W., BREER, H., and HARTKEMEYER, M. (1981): Local and temporal pattern of Con A - binding site aggregation during conjugation in *Euplotes vannus* (Ciliophora, Hypotrichida). J. Protozool. **28** 414-416.

LUTHE, N., and PLATTNER, H. (1986): Lectin binding sites in *Paramecium tetraurelia* cells. II Labeling analysis predominantly of non-secretory components. Histochemistry **85** : 377-388.

LUTHE, N., PLATTNER, H., HAAKE, B., WALTHER, P., and MULLER, M. (1986): Lectin binding sites in *Paramecium tetraurelia* cells. I. Labeling analysis predominantly of secretory components. Histochemistry **85** : 365-376.

MALONEY, M.S. (1984): Phytohemagglutinin induces delays in oral regeneration in the ciliate *Stentor coeruleus*. J. Protozool. **31**: 170-174.

- (1986): Concanavalin A inhibits oral regeneration in *Stentor coeruleus* through a mechanism involving binding to the cell surface. J. Cell Physiol. **128**: 113-120.

- (1988): Con A binds to the membranellar and somatic cilia of *Stentor* and to the developing oral primordium during oral regeneration. J. Protozool. **35**: 502-505.

MERKEL, S.J., KANESHIRO, E.S., and GRUENSTEIN, E.I. (1981): Characterization of the cilia and ciliary membrane proteins of wild-type *Paramecium tetraaurelia* and a pawn mutant. J. Cell Biol. **89**: 206-215.

PAGE, F.C.(1983): Marine Gymnamoebae. Institute of Terrestrial Ecology, Cambridge.

PAGE, F.C. (1987). The classification of 'naked' amoebae (Phylum Rhizopoda). Arch. Protistenkd. **133**: 199-217.

POLNE-FULLER, M. (1987): A multinucleated marine amoeba which digests seaweeds. J. Protozool. **34**: 159-165.

PRESTON, T.M., O'DELL, D.S., and KING, C.A. (1975). Fluorescence microscope observations of some surface components of the amoebaflagellate *Naegleria gruberi* during amoeboid locomotion. Cytobios **207**: 207-216.

ROGERSON, A. (1988). DAPI-staining for the rapid examination of nuclei and parasomes in marine gymnamoebae. Arch. Protistenkd. **135**: 289-298.

ROGERSON, A., POLNE-FULLER, M., TRENCH, R.K., and GIBOR, A. (1990). A laboratory induced symbiosis between a marine amoeba, *Trichosphaerium*, and the dinoflagellate, *Symbiodinium*. Symbiosis (in press).

SHARON, N., REISNER, Y., RAVID, A., and PRUJANSKY, A. (1979): Studies on the interaction of lectins with polysaccharides on lymphocyte cell surfaces. In: GOLDSTEIN, I.J., (ed), Carbohydrate-protein interaction. pp. 1-11. American Chemical Society, Washington, D.C.

TORRES, C., and HART, G.W. (1984): Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. J. Biol. Chem. **259**: 3308-3317.

WATANABE, S., TOYOHARA, A., SUZAKI, T., and SHIGENAKA, Y. (1981): The relation of concanavalin A receptor distribution to the conjugation process in *Tetrahymena thermophila*. J. Protozool. **28**: 171-175.

WEIR, D.M. (1980): Surface carbohydrates and lectins in cellular recognition. Immunol. Today 1 :45-51.

Figure Legends

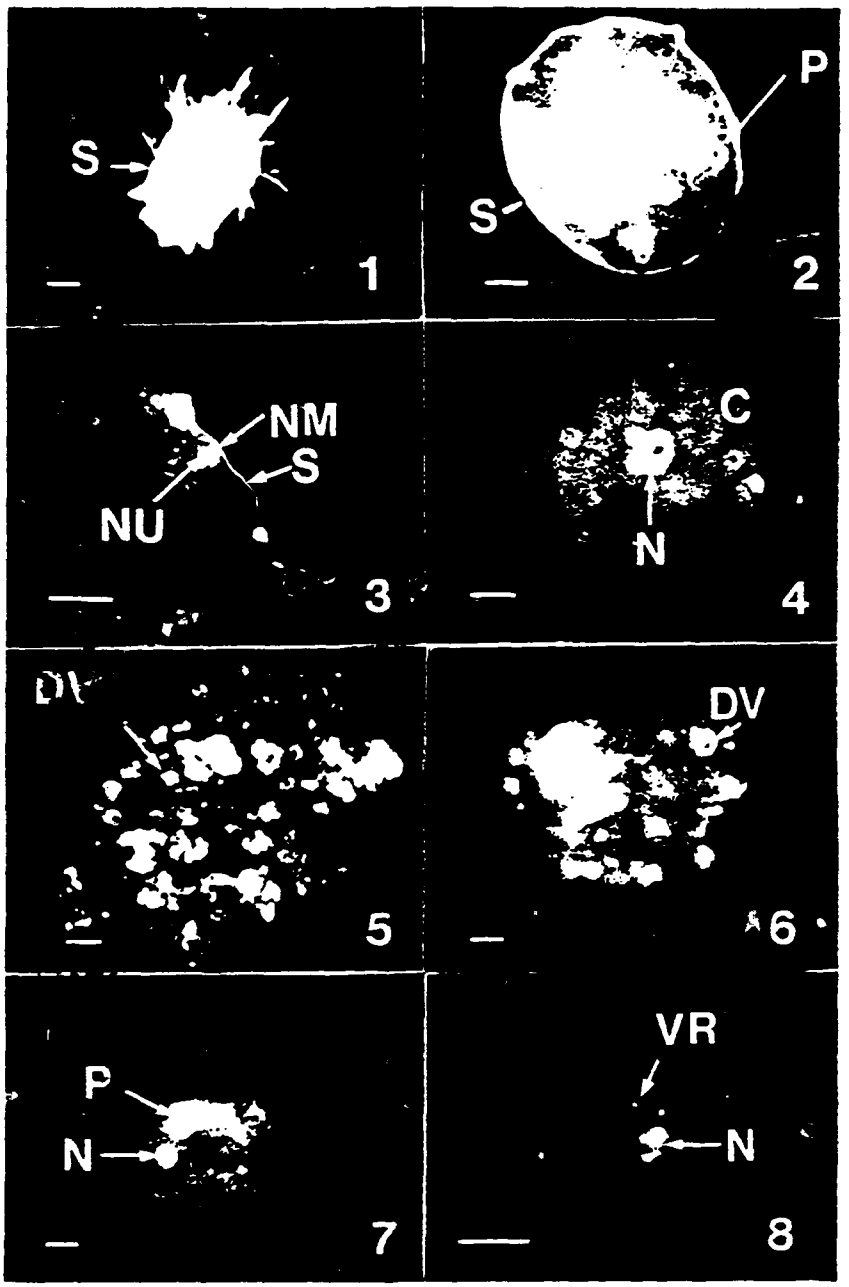
Figs. 1-8. Fluorescent micrographs illustrating the 8 FITC-lectin binding sites in the amoebae shown in Table 1. Bar = 10 μm throughout. 1. *Paramoeba eilhardi* treated with Con-A. Cell surface (S). 2. *Trichosphaerium* AM-I-7 treated with Con-A. Cell surface (S); pore (P). 3. *Mayorella kuwaitensis* treated with WGA. Nuclear membrane (NM); nucleolus (NU). 4. *P. eilhardi* treated with DBA. Nucleus (N); cytoplasm (C). 5-6. *Trichosphaerium* AM-I-7 treated with WGA. Digestive vacuoles (DV), delineated by the vacuole contents in Fig 5 and by the vacuole membrane in Fig 6. 7. *P. eilhardi* treated with WGA. Nucleus (N); parasome (P). 8. *Paramoeba* HB treated with WGA. Nucleus (N); vesicular region (VR).

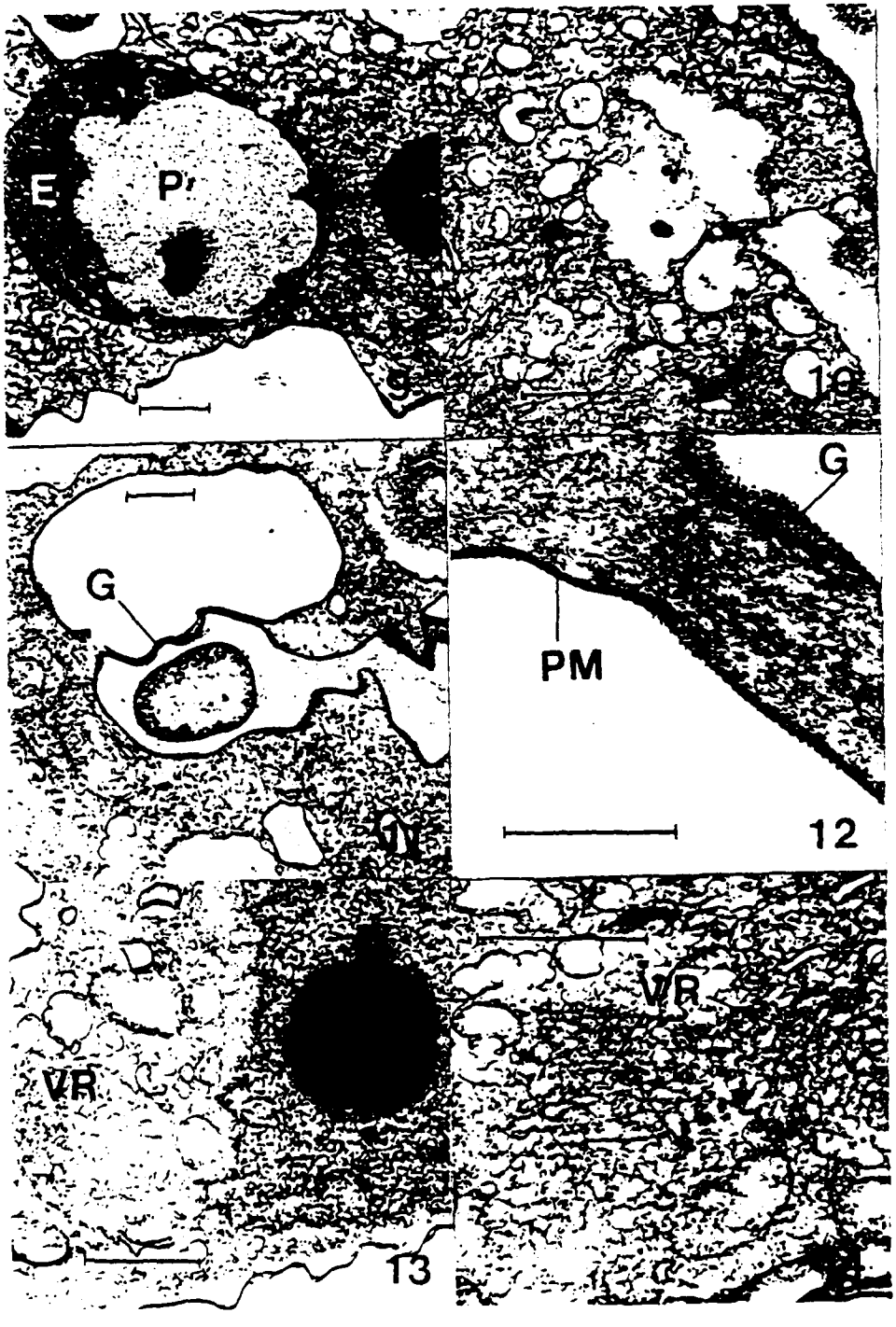
Figs. 9-14. Electron micrographs of *Paramoeba* HB. Bar = 1.0 μm except for Fig. 12 where bar = 0.5 μm. 9. Parasome (P) closely apposed to the nucleus. No connections were observed between the parasome and nucleus. Parasomes had two electron dense poles (E); only one shown in this oblique section. 10-11. Cytoplasmic regions with numerous vacuoles and vesicles. Note the angular appearance of recently endocytosed vacuoles (i.e. those with an intact glycocalyx, G). 12. Surface of amoeba with plasma membrane (PM) and glycocalyx (G) composed of tubular elements. 13-14. Cytoplasmic region adjacent to the nucleus showing the vesicular regions (VR), often found in pairs.

TABLE 1: Comparison of lectin-binding sites in 8 species of marine amoebae.

Species	lectins						
	UEA-1	Con A	RCA120	SBA	DBA	WGA	PNA
Mayorella kuwaitensis	S,N	S	DV, C N	-	-	S,NU NM,DV	-
Mayorella gemmifera	-	S	NU,C	-	C,N	NU,NM C	
Trichosphaerium (AM-1-7)	-	S	DV	-	-	DV	-
Paramoeba aestuarina	S or C	S N,P	-	S	S	S or C N,NU	S or C
Paramoeba eilhardi	C,N	S,NM P	-	-	C,N DV	S or C DV N	-
Paramoeba invadens	C	S,NM	-	C	S or C NM, NU	S or C NM,NU	S or C
Paramoeba (HB)	-	S NM	-	-	-	NM,NU VR	-
Paramoeba pemaquidensis	S or C N	S,NM	-	C	C	N,NU DV	C,N

- = no binding; S = cell surface; DV = digestive vacuole;
C = cytoplasm; N = nucleus; NM = nuclear membrane;
NU = nucleolus; P = parasome; VR = vesicular region.


S
1
P
S
2
NM
S
NU
3
C
N
4
DV
5
DV
6
P
N
7
VR
N
8


E
Pr
10
G
PM
12
G
VR
VR
13

APPENDIX III

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A Laboratory-Induced Association Between the Marine Amoeba *Trichosphaerium* AM-I-7 and The Dinoflagellate *Symbiodinium* #8

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Abstract

A laboratory-induced association between the marine amoeba *Trichosphaerium* and the dinoflagellate *Symbiodinium*, originally isolated from the anemone *Aiptasia pulchella* was examined. When cultured with algae, individual amoebae incorporated approximately 260 algae within 3 weeks partitioning them intracellularly within vacuoles. Once established, the association survived for over 2 years in culture. When dinoflagellates were abundant in the medium, the apparent turnover time of the algal population was close to 30 hr, but when algae were excluded from the medium amoebae retained a stable complement of intracellular algae. Typically, about 97.8% of the dinoflagellates phagocytosed by amoebae in the light remained viable for at least one week; while in the dark only 82.2% of the algae were viable. The transfer of photosynthetic products from alga to amoeba was 9.8% of total ^{14}C-labelled photosynthate. This may account for the slightly shorter generation times of amoebae with algae grown in the light (49.8 hr) versus those in the dark (54.6 hr). The potential for using this novel laboratory system to investigate algal/invertebrate associations is discussed.

Keywords: association, dinoflagellate, marine amoeba, *Symbiodinium*, symbiosis, *Trichosphaerium*

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1. Introduction

Trichosphaerium is a common genus of marine amoeba comprising three named species (Page, 1983). It is an unusual amoebae in that it resembles a naked form under the light microscope but in high osmoticum medium and at the EM level, a thin flexible outer test can be seen either with or without calcareous spicules. The test has many pores through which cytoplasmic protrusions, or dactylopodia, project and it is these distinctive pseudopodia which facilitate the identification of the genus *Trichosphaerium*.

Trichosphaerium has been frequently isolated by the authors and others (Angell, 1975, 1976; Page, 1983) from neritic regions throughout the world but these isolates have never been reported to harbor symbiontic algae.

Among the marine Sarcodina, those protists bearing pseudopodia of one form or another, only the foraminiferans (Lee, 1983; Lee and McEnery, 1983), radiolarians (Anderson, 1983) and acantharians (Febvre and Febvre-Chevalier, 1979) have been shown to sequester algal symbionts. In the case of foraminiferans, the retention of symbionts is a very common phenomenon and they incorporate a broad range of algal types including dinoflagellates, chlorophyceans, diatoms and a unicellular rhodophyte (reviewed in Smith and Douglas, 1988). Moreover, several marine protists, notably oligotrich ciliates and foraminiferans, have been shown to sequester the chloroplasts of the algae upon which they feed (Laval-Peuto and Febvre, 1986; Lee and Lanners, 1988).

While the aforementioned symbiotic associations are relatively common in the marine plankton, they do not lend themselves to experimentation as few of the protozoan hosts can be successfully cultivated in the laboratory. *Trichosphaerium*, on the other hand, grows with ease in the laboratory on a bacterial diet or on a variety of micro- and macro- algae (Poine-Fuller, 1987). During experiments testing for suitable food sources it was found that this amoeba readily sequestered, rather than digested cells of the symbiotic dinoflagellate *Symbiodinium* sp. #8.

It is generally accepted that there are complex recognition processes involved in the establishment of associations between microalgae and invertebrates (Trench, 1988). However, little is known about the mechanisms controlling this phenomenon. *Trichosphaerium*, in association with its dinoflagellate symbionts, may provide a unique model for future studies on protozoan/algal symbiosis and in a broader context a model for studying cell-cell recognition. Towards this end, we present a detailed description of this novel laboratory-induced association.

. Materials and Methods

'ulture

Trichosphaerium AM-I-7 was isolated from the brown alga *Sargassum mucum*, collected at Alegria Beach, Santa Barbara County, CA. Full details ı culture and isolation procedures for *Trichosphaerium* are given elsewhere ?oine-Fuller, 1987). Stock cultures of axenic amoebae were maintained on round and autoclaved algae in PES enriched seawater (Provasoli, 1968). moebae were also cultured on bacteria in Cerophyl/seawater (C7medium ?age, 1983). The dinoflagellate *Symbiodinium* sp.#8 was originally isolated om the sea anemone *Aiptasia pulchella*. It was maintained in axenic culture ı ASPSA (Ahles, 1967).

'stablishment of association

Three methods were used to establish the amoebal/algal laboratory asociation. The first method mixed axenically reared *Symbiodinium* and *'richosphaerium* in PES medium. The two other methods had bacterial prey resent promoting amoebal replication. One of the bacterized procedures sed organically rich C75S medium which encouraged luxuriant bacterial rowth. The other method used PES artificial medium which, because of s low organic content supported only a background bacterial population. ı all cases the media supported dinoflagellate growth in the light. When *'richosphaerium* were added, they ingested, partitioned and sequestered alae. The fastest uptake rates of the algae by the amoebae occured at the acteria-free and low bacterial cultures. In these culture systems, and in all ıbsequent experiments (unless noted) the concentration of dinoflagellates 'ee on the bottom of the culture vessel was approximately 750 cells mm^{-2}.

'ptake rate of dinoflagellates by by amoebae

For reasons of convenience the incorporation of algae by amoebae was examined in bacterized cultures using PES and C75S media. The number of lgal cells incorporated over time was determined by counting the number of lgae in 25 randomly selected amoebae. In the early stages, when amoebae arbored few algae, cells were enumerated directly on an inverted microscope. ater, when amoebae contained many symbionts, cells were compressed uner coverslips and algae counted at higher magnification.

Determination of growth rate

Individual amoebae were micropipetted into 0.5 ml aliquots of media in cavity slides. To prevent evaporation and contamination, a surface layer of sterile paraffin oil was added. Conditions in each experiment varied. Some amoebae were full of symbiotic algae while others were taken from cultures fed on bacteria only. Two different media were used for comparison (C75S and PES) and experiments were conducted with or without algae in the light and in the dark. For each experiment, 15 replicates were set up and the number of amoebae counted at least twice daily. Semilog regressions of cell count against time (hr) were computed for the exponential phase of growth.

Fate of ingested algae

The nature of the vacuoles with dinoflagellates, (whether they were digestive or perialgal) was examined by neutral red staining and by lectin binding. Neutral red stains food vacuoles of amoebae from red to yellow depending upon the pH of the vacuoles (Finlay et al., 1988). Free dinoflagellates in the media were examined also for stain accumulation. This gave information about the proportion of viable and non-viable algae in the media. Dead cells with completely or partially degraded cell walls stained dark red. At least 1000 algal cells, sequestered or free, were counted on each experimental occasion.

The lectins *Ricinus communis* agglutinin (RCA^{120}) and *Triticum vulgaris* agglutinin (WGA) bind to the digestive vacuole membrane and digestive vacuole contents of *Trichosphaerium* respectively (unpublished). Lectins conjugated with FITC, were purchased from Vector Laboratories (Burlingame, CA). For staining, amoebae were fixed in 8% formaldehyde, washed in PES medium and treated for 30 min with 40 μg of lectin in 1 ml of medium. After three further washes, cells were examined in a Zeiss Photomicroscope III with epifluorescence.

Algal divisions within Trichosphaerium *and estimated retention time*

Individual *Trichosphaerium* packed with algae were compressed under a cover glass and examined at 1000× magnification. The number of dividing and non-dividing algae in 20 amoebae were counted directly as were an equivalent number of algae free in the medium. Thin sections were examined by TEM for evidence of dividing intracellular dinoflagellates. Procedures for specimen preparation are given below.

To estimate the rate of flux of algae through amoebae, several complementary methods were used. Individual amoebae in cultures containing free dinoflagellates were examined in an inverted microscope with low illumination for 30 min intervals (n=20). Over this period, the number of algae ingested and egested by *Trichosphaerium* was counted. Amoebae were also observed for longer time intervals using time-lapse photography. These experiments lasted 12–48 hr and photographs were recorded every 2 min. No external free dinoflagellates were present in these experiments. Because of visual limitation due to the orientation of large numbers of algae inside an amoeba, only amoebae with 10 or less sequestered algae were examined; higher numbers of intracellular algae were impossible to monitor automatically. The final method to investigate flux rate used dinoflagellates labelled with the electron dense marker, cationized ferritin (Sigma Chemical Co., St. Louis, MO). This label readily bound to the cell surface of *Symbiodinium* when used at a concentration of 50 μg ml^{-1}. After 24 hr of exposure to labelled algae, amoebae were fixed, thin sectioned and examined by TEM. The percentage of intracellularly labelled algae (i.e. those ingested within 24 hr) to non-labelled algae was calculated.

Electron microscopy

Amoebae were fixed simultaneously in 2% glutaraldehyde and 1% osmium tetroxide in a 1:1 mixture of C75S medium and 0.1 M sodium cacodylate buffer (pH 7.2), at 4°C for 30 min. After 4 washes in cold distilled water, cells were dehydrated through an acetone series and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 TEM. Sections containing ferritin labelled cells were left unstained.

Transfer of photosynthetic product from algae to amoebae

Amoebae packed with algae were incubated for 1 hr in the light in 5 ml C75S medium containing ^{14}C sodium bicarbonate (total activity, 5 μC). The number of amoebae was determined in a parallel sample by subsampling and counting the cells in 10 μl drops (n=10). After the 1 hr incubation, amoebae were washed 8 times in PES medium. One sample was concentrated by centrifugation, added to 5 ml of Hydrofluor (National Diagnostics, Somerville, NJ) and counted by liquid scintillation spectrometry. This gave a measure of the total incorporation of label by dinoflagellates. A second sample was washed in PES and placed in the dark for 18 hr. When *Trichosphaerium*

is washed in inorganic seawater medium (PES), the cells undergo multiple fission typically dividing into a large number of small (20 μm) cells. During the process of fission cells eject all their vacuolar content thereby providing a convenient method for separating the algal and amoebal components. After 18 hr, released algae were decanted leaving the amoebae adhering to the surface of the culture vessel. Both samples (amoebae and algae) were collected separately, rinsed in fresh media, pelleted by centrifugation and counted by scintillation counter. Controls used amoebae incubated with label in the dark and free dinoflagellates in labelled medium in the light. All translocation experiments were repeated three times.

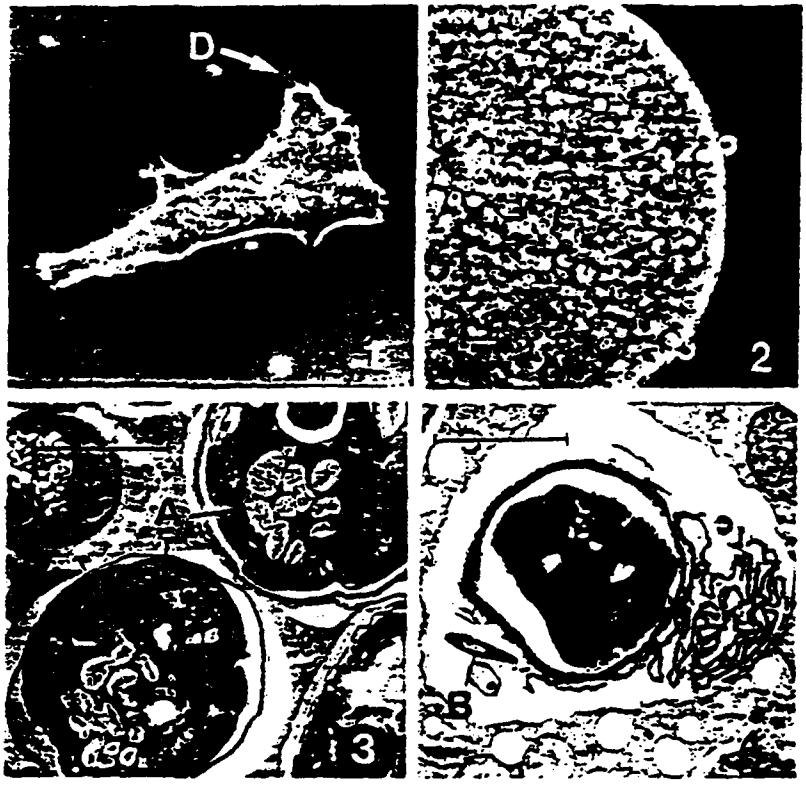
The transfer of labelled photosynthetic product to amoebae was verified by autoradiography. Procedures were as described but instead of scintillation counting, amoebae were air-dried on slides and coated with Kodak NTB-2 nuclear track emulsion. After 10 days, the emulsion was developed for 2 min (20°C) in undiluted Kodak D 19 and the number of silver grains overlying whole cells and background areas compared.

3. Results

The laboratory association between the amoeba, *Trichosphaerium* AM-I-7 (without algae, Fig. 1) and the dinoflagellate, *Symbiodinium* sp. #8 survived for over 2 years in bacteria free PES with no change of medium. In C75S medium dense populations of bacteria took over the cultures killing the amoebae.

In a mature association single amoebae sequestered over 100 algal cells and commonly 260 + 49 S.D. (n=20) dinoflagellates per amoeba (Fig. 2, and front cover picture). The establishment of this intracellular algal population was as follows: after an initial lag phase (up to 100 hr) in which uptake rates of algae were low (typically 5 cells in total) the rate of incorporation of algae increased exponentially. The semilogarithmic plot describing this phase had the equation $y = 0.12x - 0.418 (r = 0.987)$. Thus amoebae sequestered algae at a rate of about 1 cell h-1 up to a saturation level of about 260 algae. This rate is similar to the rate of incorporation of "inert" plastic beads (15 μm diameter). Here the slope describing the exponential phase was 0.011 (r = 0.901). [*Note:* "insert"= A mutant of *Trichosphaerium Am.-I-7* (presently under investigation), is capable of degrading and digesting plastic polymers such as polyethelenes and polyvinyls. Therefore "inert" plastic beads may be recognized by *Trichosphaerium* as potential food source.]

The vast majority of sequestered dinoflagellates were maintained intracellularly as viable cells. Examination of algae by TEM showed that the



Figures 1–4. (1) LM of *Trichosphaerium* fed bacteria. Note the dactylopodia (D) which protrude through pores in the amoeba test. Bar = 10 μm. (2) LM of *Trichosphaerium* partially compressed to show numerous intracellular dinoflagellates. Bar = 10 μm. (3) TEM of *Trichosphaerium* showing intact intracellular algae (A). Bar = 5 μm. (4) TEM of *Trichosphaerium* with dinoflagellate in food vacuole showing evidence of digestion. Algae (B) also in vacuole. Bar = 5 μm.

majority were structurally intact (Fig. 3) although occasionally partially digested or autolysed cells in vacuoles were observed (Fig. 4). This result was supported by staining with FITC-conjugated lectins specific for digestive vacuoles. Vacuoles full of digestible cells (yeast, *Chlamydomonas*, ground macroalgae, etc.) stained brightly by the lectins RCA^{120} and GWA while lectin treatments on amoebae packed with *Symbiodinium* #8 failed to stain the membranes or the contents of vacuoles.

The proportion of both free and sequestered algae undergoing digestion or

Table 1. Percentage of dinoflagellates accumulating neutral red stain (i.e. non-viable cells) and percentage of algae undergoing cell division. Observations made 20 days after inoculation into PES media.

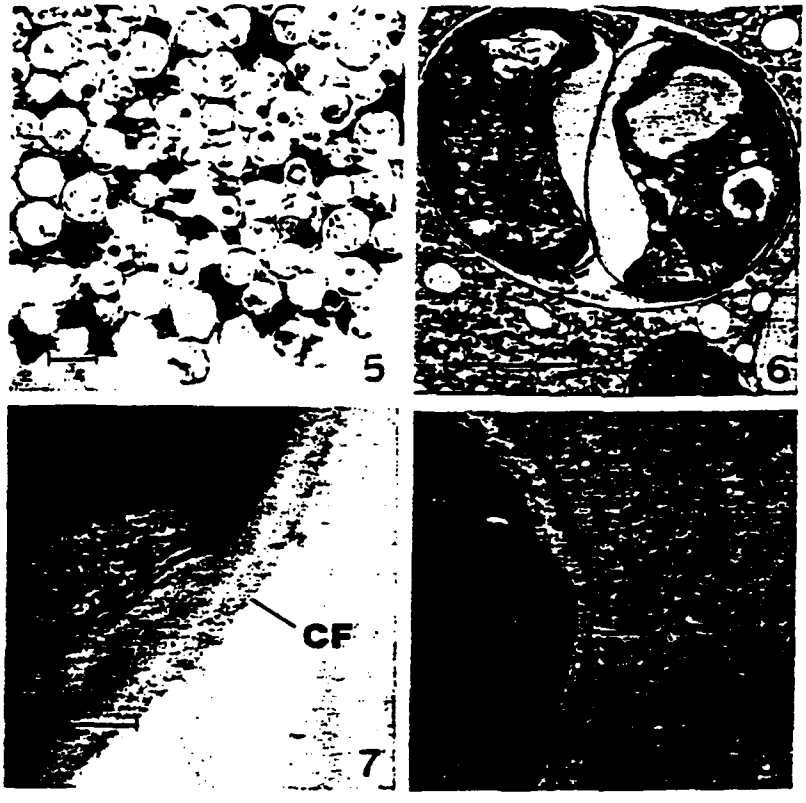
Experimental condition	Light/Dark	Percentage[1] Staining	Dividing
Symbiodinium free	L	0.1	6.5
No amoebae	D	0.2	2.6
Symbiodinium free	L	0.5	4.9
With amoebae	D	7.2	0.1
Symbiodinium sequestered	L	2.2	5.2
Within amoebae	D	17.8	0.4

[1] Percentages based on at least 1000 observations

possibly autolysis was estimated by staining with neutral red (Table 1 and Fig. 5). *Symbiodinium* free in the medium were generally intact with only 0.1–0.5% taking up stain when cultures were kept in the light. However, in the dark 7.2% of free dinoflagellates showed staining. The majority of algae sequestered by amoebae in the light were viable (only 2.2% stained). In the dark, however, many more intracellular algae showed evidence of digestion or autolysis with 17.8% taking up neutral red.

Sequestered algae showed some evidence of replication within amoebae. From examination of thin sections by TEM, dividing cells were occasionally observed over a 24 hr period (Fig. 6). However, comparisons between the number of dividing intracellular algae and dividing free algae (Table 1) did not show any significant differences.

The observed low rate of intracellular replication can be related to the flux rate, and low residence time, of most of the algae within the host amoebae. Using cationized ferritin as a marker to identify freshly sequestered dinoflagellates (Figs. 7 and 8), it was found that 90% of the algae were ingested within a 24 hr period when amoebae were in culture with ample free dinoflagellates. Direct observations support this result. During a series of 30 min observations (n=20) the mean rate of uptake was 10 cells hr-1 with the release of a similar number. This is higher than the rate of uptake by cells during establishment of the association. This rate, however, implies a significant daily turnover in line with that indicated by ferritin labeling. When similar observations were made on cells in media with no free dinoflagellates, much lower flux rates were found. Here amoebae retained a stable resident population of algae over the series of 12 hr observational periods. However, these amoebae contained on average, only 15 dinoflagellates.



Figures 5–8. (5) Sequestered dinoflagellates of *Trichosphaerium* treated with neutral red to stain partially digested vacuoles (in black). Bar = 10 μm. (6) TEM of *Trichosphaerium* showing recently divided intracellular alga. Amoebal cytoplasm (C). Bar = 5 μm. (7) TEM (unstained section) showing part of a wall of a free dinoflagellate to which cationized ferritin was bound (CF). Bar = 0.2 μm. (8) TEM (unstained section) showing edge of recently ingested dinoflagellate identified by its cationized ferritin (CF) labeled wall. Amoeba cytoplasm (C). Bar = 0.2 μm.

Growth rates of amoebae under different experimental conditions were computed from semilogarthimic plots (base 10) of the exponential phase describing cell counts with time. These rates, and corresponding generation times are given in Table 2. Amoebae packed with algae and cultured in PES with ample free dinoflagellates in the surrounding medium divided after 49.8 hr in the light and 54.6 hr in the dark. The generation time of amoebae in PES medium without free dinoflagellates was 327 hr in the light; cell division was completely suppressed in the dark. *Trichosphaerium* grown

Table 2. Comparison of growth rates and calculated generation times of *Trichosphaerium* both with and without intracellular *Symbiodinium*. The effects of different media, light, dark and whether free dinoflagellates were added to the culture system were examined.

Pre-experimental condition	Culture medium	Light/ dark	With/ without algae	Specific[1] growth rate (hr-1)	generation time (hr)
Amoebae full of sequestered dinoflagellates	PES	L	+	6.0 (1.8)	49.8
	PES	D	+	5.5 (1.7)	54.6
	PES	L	-	0.9 (0.4)	327
	PES	D	-	no growth	-
Amoebae full of sequestered dinoflagellates	C75S	L	-	15.5 (4.0)	19.3
	C75S	D	-	8.9 (2.0)	22.7
Amoebae without dinoflagellates (bacterial diet)	C75S	L	-	11.9 (2.5)	25.2

[1] Specific growth rates ($\times 10^{-3}$); all regressions with correlation coefficients (r) 0.9; standard deviations in parenthesis

with ample bacteria in C75S medium either with or without sequestered dinoflagellates grew rapidly and divided in 24 hr or less.

The percentage of total ^{14}C labelled product transferred from algae to amoebae was 9.8% (+5.0 S.D.). It is unlikely that any of this labelled product was due to digestion of dinoflagellates since amoebae induced to undergo multiple fission rapidly expel their vacuolar contents within the first few hours. Moreover, the physiological changes which accompany this phenomenon are not conducive to digestion and assimilation of ingesta. Algae released from amoebae harbored 25.9% (+9.9 S.D.) of C^{-14} label, the remainder 64.1% was unaccounted for. It could have been rinsed away during the multiple fission as decomposed but not yet assimilated digestive products. Autoradiography on small amoebae (after fission) supported the translocation of photosynthetic product. Counts of silver grains in an area overlying amoebae ($\approx$ 400 μm^2) averaged 140.8 + 39.6 S.D. (n=15) which was significantly above that of background (15.8 + 3.8 S.D.).

4. Discussion

Many symbiotic associations involving protists and algae have been described (reviewed in Smith and Douglas, 1988) with the vast majority involving dinoflagellates of the genus *Symbiodinium*. This is also true for sarcodines

where planktonic foraminiferans, radiolarians and acantharians frequently associate with dinoflagellates (Lee et al., 1985). Despite the fact that research on marine protozoa has increased markedly, largely because of an increased awareness about the ecological importance of symbiosis, no algal symbionts have been reported from any species of *Trichosphaerium* nor from any of the morphologically similar gymnamoebae. This suggests that the association between *Trichosphaerium* and *Symbiodinium* described in this paper is a laboratory phenomenon and is either rare or does nor occur in the marine environment. In support of this, all attempts by the authors to isolate dinoflagellate-laden cells from the field were unsuccessful.

In view of what we understand about protist/algal symbioses, this is not surprising. The majority of associations described in sarcodines and ciliates occur in the euphotic zone of oligotrophic waters. In this nutrient depleted environment it is beneficial for the host to harbor endosymbiotic algae and profit from the retention and recycling of nitrogen and phosphorous compounds within an essentially closed system. *Trichosphaerium*, on the other hand, is common in littoral habitats where it grazes on the bacteria and microalgae associated with the surfaces of seaweeds and on the algal tissue itself (Polne-Fuller, 1987). In this nutrient-rich microhabitat the retention of symbionts is not favored from an energetic standpoint.

Our laboratory results demonstrate the importance of nutrient status on the stability of the algal association. When amoebae containing algae were cultured in organically rich bacterized media they egested over 95% of their dinoflagellates within 24 hr and preyed on bacteria. Under these conditions, *Trichosphaerium* reproduced rapidly, dividing every 20 hr or less. At the other extreme, when amoebae with algae were cultured in inorganic medium with few bacteria and no free algae they retained their full complement of dinoflagellates. Their growth rates were low and they divided only once every 2 weeks or longer. A similar retention of symbionts in inorganic media was observed by time-lapse photomicroscopy where amoebae in cultures without free dinoflagellates retained a stable complement of symbionts. Under these conditions, algae were retained intracellularly without flux and the association approached that of a true symbiosis.

The stability and duration of the association was different when dinoflagellates were abundant in the inorganic medium. Here, *Trichosphaerium* turned over the majority of its algal population in about 30 hr. For these reasons we prefer, at this time, to term the relationship an "induced association" rather than a symbiosis even though other features of the relationship may imply mutualism. For example, amoebae retained a full complement

of intracellular algae, albeit a fluxing population, for over 2 years in culture. Furthermore, although *Trichosphaerium* was not nutritionally dependent upon its sequestered algae, feeding instead on bacteria and perhaps a small percentage of dinoflagellates, they did assimilate 10% of the photosynthate derived from their associated algae. This supplementary source of nutrition may have accounted for the slightly improved growth of amoebae with algae in the light compared to those in the dark. There is little information on the nutritional relationships of other sarcodine/algal associations, but there are indications that photosynthetic products alone rarely support rapid growth. For example, the foraminiferan *Globigerinoide sacculifer* grows slowly but fails to reproduce when starved in the light (Lee and McEnery, 1983). It appears, therefore, that one of the major benefits of retaining symbionts is to ensure survival of the host in habitats which are subject to periodic fluctuations in prey and/or nutrients.

The laboratory induced association described here has several advantages for the study of algal/invertebrate symbioses. Aside from the fact that both partners can be cultured with ease, *Trichosphaerium* can readily be induced to egest its intracellular algae upon transfer to unenriched seawater where amoebae undergo multiple fission. While this sensitivity on the part of the amoebae can be problematic during routine handling, the benefits outweigh the disadvantages as intracellular algae can easily be separated from host amoebae. Moreover, *Trichosphaerium* has the ability to discriminate between different dinoflagellate species. In a separate study (manuscript in prep.) it was shown that the incorporation of *Symbiodinium* sp. #8 is favored by this amoeba over other species of symbiotic dinoflagellates and thus supports the presently established fact that symbiosis between microalgae and invertebrates demonstrate specificity (Trench, 1987, 1988). In light of the above, perhaps the greatest promise for this system is as a tool to investigate the mechanisms of cell recognition. These are the processes by which algae first gain entrance into the cell and the subsequent processes that determine whether or not these algae will be retained by the host. We hope that further studies with the *Trichosphaerium/Symbiodinium* association may solve some of the many unanswered questions surrounding these processes.

Acknowledgement

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REFERENCES

Ahles, M.D 1967. Some aspects of the morphology and physiology of *Symbiodinium microadriaticum*. Ph.D. thesis, Fordham University, NY.

Anderson, O.R. 1983. The radiolarian symbiosis. In: *Algal Symbiosis*. L.J. Goff, ed. Cambridge University Press, Cambridge, pp. 69–89.

Angell, R.W. 1975. Structure of *Trichosphaerium micrum* sp. n. *J. Protozool.* 22: 18–22.

Angell, R.W. 1976. Observations on *Trichosphaerium platyrum* sp. n. *J. Protozool.* 23: 357–364.

Febvre, J. and Febvre-Chevalier, C. 1979. Ultrastructural study of zooxanthellae of the three species of acantharia (Protozoa: Actinopoda), with details of their taxonomic position in the Prymnesiales (Prymnesiophyceae, Hibberd, 1976). *J. Mar. Biol. Assoc. U.K.* 59: 215–226.

Finlay, B.J., Rogerson, A., and Cowling, A.J. 1988. *A Beginners Guide to the Collection, Isolation, Cultivation and Identification of Freshwater Protozoa*. CCAP Publication, Ambleside, U.K.

Laval-Peuto, M. and Febvre, M. 1986. On plastid symbiosis in *Tontonia appendiculariformis* (Ciliophora, Oligotrichina). *Biosystems* 19: 137–158.

Lee, J.J. 1983. Perspectives on algal endosymbionts in larger foraminifera. *Int. Rev. Cytol. Suppl.* 14: 49–77.

Lee, J.J. and McEnery, M. 1983. Symbiosis in foraminifera. In: *Algal Symbiosis*. L.J. Goff, ed. Cambridge University Press, Cambridge, pp. 37–68.

Lee, J.J., Soldo, A.T., Reisser, W., Lee, M.J., Jeon, K.W., and Gortz, H-D. 1985. The extent of algal and bacterial endosymbioses in protozoa. *J. Protozool.* 32: 391–403.

Lee, J.J. and Lanners, E. 1988. The retention of chloroplasts by the foraminifer *Elphidium crispum*. *Symbiosis* 5: 45–60.

Page, F.C. 1983. *Marine Gymnamoebae*. Cambridge Inst. of Terrestrial Ecology, Cambridge, U.K.

Poine-Fuller, M. 1987. A multinucleate marine amoeba which digests seaweeds. *J. Protozool.* 34: 159–165.

Provasoli, L. 1968. Media and prospects for cultivation of marine algae. In: *Cultures and Collections of Algae*. A. Watanabe and A. Hattori, eds. Proc. U.S.-Japan Conf. Hakone, Sept. 1966, Jpn. Soc. Plant Physiol., Tokyo, pp. 63–75.

Smith, D.C. and Douglas, A.E. 1987. *The Biology of Symbiosis*. Edward Arnold, London.

Trench, R.K. 1987. Dinoflagellates in non-parasitic symbioses. In: *The Biology on Dinoflagellates*. F.J.R. Taylor, ed. Blackwell, Oxford, pp. 531–570.

Trench, R.K. 1988. Specificity in dinomastigote-marine invertebrate symbioses: An evaluation of hypotheses of mechanisms involved in producing specificity. In: *Cell to Cell Signals in Plant, Animal and Microbial Symbiosis*. S. Scannerini et al., eds. NATO ASI Series, Vol. H17, Springer-Verlag, Berlin, pp. 325–346.

APPENDIX IV

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NOTE

A NOVEL TECHNIQUE FOR PREPARATION OF AXENIC CULTURES OF *SYMBIODINIUM* (PYRROPHYTA) THROUGH SELECTIVE DIGESTION BY AMOEBAE[1]

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ABSTRACT

The marine amoeba Trichosphaerium *Am-I-7 was used as a tool for preparing unialgal axenic cultures of nondigestible* Symbiodinium *and* Porphyridium *species. The resistance of these unicellular algae to the amoebal digestive enzymes, and the differential digestion of bacteria, protozoans, and other algae, resulted in cleansed cells of* Symbiodinium *and* Porphyridium *that remained in the amoebal food vacuoles. During multiple fission, the amoeba evacuated its food vacuoles and released the trapped and intact algae, which were then successfully cultured. This method of cleaning was especially useful with algal species that were sensitive to antibiotics or other germicidal agents.*

Key index words: amoeba; axenic algae; multiple fission; Porphyridium; *selective digestion;* Symbiodinium; Trichosphaerium *Am-I-7;* zooxanthellae

Axenic cultures of unicellular algae have usually been prepared by single cell isolation and rinsing or by treatments with antibiotics and other germicides (Fries 1963, 1977, Mooney and Van Staden 1985, Polne-Fuller and Gibor 1987a, b). Single cell isolation and washing of microscopic algae is a tedious process that produces few clean, viable cells. Moreover, the single cell isolation technique is rendered useless if the contaminating organisms are attached to the algal surfaces. Chemical treatments are more efficient in producing clean cells; however, some algae are sensitive to the array of chemicals that are necessary to eliminate contamination. Due to these limitations various species of zooxanthellae have not been cultured free of bacteria and other contaminants, such as a small flagellate that is often present. The importance of axenic cultures of zooxanthellae can not be overemphasized in studies of symbiotic interactions between the plant cells and their animal hosts.

The amoeba *Trichosphaerium* is a large (15–2000 μm), omnivorous marine organism that digests a variety of seaweeds and algae (Polne-Fuller 1987) as well as bacteria, flagellates, and various other protozoans (unpubl. data). Some algae such as species of *Symbiodinium* are taken into amoebal cytoplasmic vacuoles but not digested (Rogerson et al. 1989). A similar phenomenon of slow, or limited, digestion also occurs with the red unicellular alga *Porphyridium.* The ability of the amoebae to differentially digest bacteria, but not the various species of *Symbiodinium* and *Porphyridium,* was used as a tool for cleaning these algae from digestible contaminating microorganisms.

Four methods were used for isolating axenic algae through the cytoplasmic vacuoles of the amoeba *Trichosphaerium* Am-I-7.

1) Antibiotics + digestion + multiple fission (xp). Amoebae were mixed with contaminated cultures of algae (amoebae: algal ratio of about 1:50). The amoebae ingested the algae along with other food particles and packed them in cytoplasmic vacuoles (Fig. 1). The culture vessels that contained the algal-packed amoebae were rinsed gently with sea water and flooded with a mixture of 10 antibiotics for 2 days (Table 1). A mixture of five antibiotics (Table 1: I) was used initially with cleaning rates of about 75–80%. Remaining contaminants (when present) were mostly two strains of bacteria, a white smooth colony and a yellow smooth colony. Five additional antibiotics (Table 1: II) that were added to the mixture increased the rate of successful axenic preparations to over 90%. The mixture of 10 antibiotics was also useful in cleaning unicellular algae, filamentous algae, seaweeds, and amoebae (Polne-Fuller et al., unpubl.). The *Trichosphaerium* remained attached to the culture vessel, which simplified the rinsing process. The algal-filled amoebae were detached from the substrate using a forced water stream produced by a pasteur pipet, transferred to a new culture vessel, and maintained in clean antibiotics for 2–3 weeks. During this period no other food source was made available to the amoebae (partial starvation period). The amoebae continued to digest the remaining digestible particles in their food vacuoles while the indigestible algae remained intact inside the cytoplasm. The indigestible algae were compartmentalized in perialgal vacuoles, whereas the edible particles were in food vacuoles (Rogerson et al. 1990).

After the partial starvation period, the amoebae were placed in fresh antibiotic mixture for 2 more days. They were rinsed four times with autoclaved sea water, dislodged from the substrate using the forced water stream produced by a pasteur pipet, and transferred to clean culture vessels where they were left to attach for 20 min. Three additional rinses induced the amoebae to go through a multiple fission (Polne-Fuller 1987, Polne-Fuller, un-

TABLE 1. *Antibiotic stock solution, 10× concentration. The solution was prepared in sea water (or fresh water) and diluted 1:10 in sea water for the amoebae and in PES medium for the algae.*

To 100 mL double distilled water (or sea water) add:

I		II	
Penicillin-G	1000 mg	Erythromycin	6 mg
Streptomycin	2000 mg	Gentamycin	8 mg
Kanamycin	1000 mg	Polymixin-B	16 mg
Neomycin	200 mg	Tetracycline	12 mg
Nystatin	15 mg	Vancomycin	12 mg

[1] Received 26 December 1990.

contaminants were attached to the amoebal cell wall. In this method, the starved amoebae containing the algae were treated with antibiotics for 2 days and flooded for 10–30 s with either a 0.5% perchloride solution in double distilled water, a 30% ethanol solution in DDW, DDW water alone, or a 10% Betadine solution in sea water. These chemical treatments killed the amoebae and the external contaminants but did not kill the algae protected inside the amoebal cytoplasmic vacuoles.

The dead but clean amoebae were collected axenically by centrifugation, rinsed twice, and teased apart manually using pulled glass pipets. For large numbers of amoebae, a small homogenizer (Biospec 1281) was used for 10 s at speed 12. A probe sonicator was also useful for breaking the amoebae with minimal damage to the algae (15 s, setting 4, on ice). The algae were collected by filtration through sterile nylon nets (20 μm) and rinsed three times with sterile 0.5% PEG in sea water. Such algal preparations still contained some amoebal membranes even after excessive rinsing, but these did not inhibit the growth or cell division of the algae.

It was necessary to allow sufficient time for the live amoebae to digest the contaminants. Depending on the contaminating organisms, starvation periods as short as 5 days (yeast, most bacteria, and most diatoms) and as long as 2 weeks (unicellular red algae, flagellates, ciliates, and fungi) were necessary to assure the elimination of digestible cells. Some diatoms and flagellates required longer periods to be digested. Several fungi developed faster than the amoebae could phagocytose and digest them, and several species of ciliates and flagellates were too active to be captured.

Of the four methods described, method #1 was usually effective in producing axenic algae. Three isolates of *Symbiodinium* from the sea anemone *Anthopleura* were successfully cleansed using the first method. However, a fourth isolate of the same species contained a bacterium resistant to the antibiotics. For this isolate the agar surface (method #3) was used successfully. A fifth isolate of the same species contained a swift flagellate that had to be eliminated using a 0.5% perchloride solution or a 30% ethanol solution for 10 s, as in method #4. Most species of *Symbiodinium* that were tested were resistant to the antibiotic mixture and were cleaned using methods #1 or #3. *Symbiodinium* #45, which turned pale in the antibiotic mixture, was cleaned by attachment to nylon netting (method #2). *Symbiodinium* #344, which released sticky polysaccharides and collected particulate matter from the medium, was cleaned as the amoebae migrated on agar, releasing algal cells in their trail (method #3). About 40% of the released algae developed axenic colonies on the agar surfaces. Method #4 was used to eliminate a fast-swimming spiral bacterium from the red unicellular alga *Porphyridium*. It was also used for an isolate of *Symbiodinium* #8 and one of the isolates of *Symbiodinium anthopleura*, which contained small swimming flagellates resistant to the antibiotics and too motile to be engulfed by the amoebae.

With these four methods isolates of four species of the symbiotic dinoflagellate *Symbiodinium* (#8, 45, 61, and 344), and the red unicellular algae *Porphoridium cinctum*, emerged viable and axenic. These methods, and combinations and variations of them, may prove useful for cleaning other algae that are not digested, or digested slowly, by various species of this and other protozoans.

The numbered *Symbiodinium* species were isolated from around the world and subcultured from the collection of symbiotic dinoflagellates of Dr. R. Trench, to whom I am grateful. The *Symbiodinium* from *Anthopleura* was isolated locally by the author. Thanks are also due to Mr. Matthew Katick for helping with the testing of the additional antibiotics, and to Dr. Aharon Gibor and Mr. Dan Coury for constructive comments on the manuscript. This work was supported in part by contract #N00014-88-K-0440 from the Office of Naval Research, and in part by contract #8-488750-25943-3 from Occidental Chemicals Inc.

Fries, L. 1963. On the cultivation of axenic red algae. *Physiol. Plant.* 16:695–708.

——— 1977. Growth regulating effects of phenylacetic acid and p-hydroxyphenylacetic acid on *Fucus spiralis* in axenic culture. *Phycologia* 16:451–5.

Mooney, P. A. & Van Staden, J. 1985. In vitro plantlet formation and multiple shoot induction in *Sargassum heterophyllum*. *S. Afr. J. Bot.* 51:41–4.

Polne-Fuller, M. 1987. A multinucleated marine amoeba which digests seaweeds. *J. Protozool.* 34:159–65.

Polne-Fuller, M. & Gibor, A. 1987a. Calluses and callus-like growth in seaweeds: induction and culture. *Hydrobiologia* 151–152:131–8.

——— 1987b. Tissue culture of seaweeds. *In* Bird, K. & Bensen, P. H. [Eds.] *Seaweed Cultivation for Renewable Resources*. Gas Research Inst., Chicago, pp. 219–40.

Provasoli, L. 1968. Media and prospects for cultivation of marine algae. *In* Watanabe, A. & Hattori, A. [Eds.] *Culture and Collections of Algae*. Japanese Society of Plant Physiologists, pp. 63–75.

Rogerson, A., Polne-Fuller, M. & Gibor, A. 1989. A laboratory induced association between the marine amoeba *Trichosphaerium Am-I-7* and the dinoflagellate *Symbiodinium #8*. *Symbiosis* 7:229–41.

——— 1990. Lectin binding sites in marine amoebae. *Arch. Protistenk.* in press.

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A NOVEL TECHNIQUE FOR PREPARATION OF AXENIC CULTURES OF SYMBIODINIUM THROUGH SELECTIVE DIGESTION BY AMOEBAE

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Running Head: Axenic cultures of Symbiodinium.

ABSTRACT

The marine amoeba Trichosphaerium Am-I-7 was used as a tool for preparing unialgal axenic cultures of nondigestible Symbiodinium and Porphyridium species. The resistance of these unicellular algae to the amoebal digestive enzymes, and the differential digestion of bacteria, protozoans, and other algae, resulted in cleansed cells of Symbiodinium and Porphyridium which remained in the amoebal food vacuoles. During multiple fission, the amoeba evacuated its food-vacuoles and released the trapped and intact algae, which were then successfully cultured. This method of cleaning was especially useful with algal species that were sensitive to antibiotics or other germicidal agents.

Key index words: amoeba; axenic algae; multiple fission; Porphyridium; selective digestion; Symbiodinium; Trichosphaerium Am-I-7; zooxanthellae;

INTRODUCTION

Axenic cultures of unicellular algae have been usually prepared by single cell isolation and rinsing, or by treatments with antibiotics and other germicides (Fries 1963, 1977, Mooney and Van Staden 1985, Polne-Fuller and Gibor 1987a,b). Single cell isolation and washing of microscopic algae is a tedious process which produces few clean viable cells. Moreover, single cell isolation technique is rendered useless if the contaminating organisms are attached to the algal surfaces. Chemical treatments are more efficient in producing clean cells; nevertheless, some algae are sensitive to the array of chemicals which are necessary to eliminate contamination. Due to these limitations various species of zooxanthellae have not been cultured free of bacteria and other contaminations such as a small flagellate which is often present. The importance of axenic cultures of zooxanthellae can not be overemphasized in studies of symbiotic interactions between the plant cells and their animal hosts.

The amoeba Trichosphaerium is a large (15-2000 um), omnivorous marine organism which digests a variety of seaweeds and algae (Polne-Fuller 1987) as well as bacteria, flagellates, and various other protozoans (unpubl. data). Some algae such as species of Symbiodinium, were taken into amoebal cytoplasmic vacuoles but were not digested (Rogerson et al. 1989). A similar phenomenon of slow, or limited, digestion also occured with the red unicellular alga Porphyridium. The ability of the amoebae to differentially digest bacteria, but not the various species of

Symbiodinium and Porphyridium, was used as a tool for cleaning these algae from digestible contaminating microorganisms.

MATERIALS AND METHODS

Four methods were used for isolating axenic algae through the cytoplasmic vacuoles of the amoeba Trichosphaerium Am-I-7: 1) Antibiotics + digestion + multiple fission (xp). Amoebae were mixed with contaminated cultures of algae (amoebae: algal ration of about 1:50) The amoebae ingested the algae along with other food particles and packed them in cytoplasmic vacuoles (Fig. 1). The culture vessels which contained the algal-packed amoebae were rinsed gently with sea water and flooded by a mixture of 10 antibiotics for two days (Table 1. I+II). A mixture of five antibiotics (Table 1. I) was used initially with cleaning rates of about 75-80%. Remaining contaminants (when present) were mostly two strains of bacteria, a white smooth colony, and a yellow smooth colony. Five additional antibiotics (Table 1. II) which were added to the mixture increased the rate of succesful axenic preparations to over 90%. The mixture of 10 antibiotics was also useful in cleaning unicellular algae, filamentous algae, seaweeds and amoebae (Polne-Fuller et al. in prep). The Trichosphaerium remained attached to the culture vessel which simplified the rinsing process. The algal-filled amoebae were detached from the substrate using a forced water stream produced by a pasteur pipet, transferred to a new culture

vessel, and maintained in clean antibiotics for 2-3 weeks. During this period no other food source was made available to the amoebae (partial-starvation period). The amoebae continued to digest the remaining digestible particles in their food vacuoles while the indigestible algae remained intact inside the cytoplasm. The indigestible algae were compartmentalized in perialgal vacuoles while the edible particles were in food vacuoles (Rogerson et al. 1990).

After the partial-starvation period, the amoebae were placed in fresh antibiotic mixture for two more days. They were rinsed four times with autoclaved sea water, disloged from the substrate using the forced water stream produced by a pasteur pipet and transferred to clean culture vessels where they were left to attach for 20 min. Three additional rinses induced the amoebae to go through a multiple fission (Polne-Fuller 1987, Polne-Fuller et al. 1990) and release the algae. The algae were centrifuged axenically (table top centrifuge, 5 min at 600 rpm), rinsed three times in sterile sea water, and cultured. A sterility test medium for marine bacteria (0.08% nutrient broth + 0.05% yeast extract + 0.01% glucose added to sea water) was used to assure sterility and select for clean cultures. This was done by plating 50 uL of algal samples on sterility test medium solidified by 1% Difco Bacto agar. The plates were incubated at 23 ^{o}C for one week. Bacterial colonies, if present, were obvious by the third to fifth day. The absence of flagellates, protozoans and other contaminating algae was determined by microscopic observations (100 to 1000X).

The clean algal colonies were subcultured axenically.

2) Release of algae through nylon net. A second set of treatments was developed with the intention of avoiding a bloom of antibiotic-resistant bacteria when the competition by the antibiotic-sensitive bacteria was eliminated. Here, amoebae containing algae were transferred to clean culture flasks, starved in sea water for two weeks, rinsed in sea water three times, flooded with antibiotics for two days, then suspended in sterile sea water containing a solution of 0.5% polyethylene-glycol (PEG, MW 20,000). PEG delayed the attachment of the amoebae to the pipets, to test tubes, and to each other. The PEG also created differential sinking conditions as the large and heavy amoebae sank after 3 min centrifugation at 400 rpm (table top centrifuge) while most of the bacteria and debris stayed in suspension. The amoebae were rinsed five times in a solution of 0.5% PEG in sterile sea water. Rinsing was done in an Eppendorf tube where roughly 1000 amoebae were suspended in 1 mL of liquid. The cells were then collected by centrifugation (600 rpm, for 5 min), rinsed twice in sterile sea water, and placed on a sterile nylon net (40 um mesh) which was glued to the bottom of a sterile plastic cup. Rinsing induced multiple fissions (Polne-Fuller et al. 1990) on the nylon netting. The whole system was kept flooded with sterile sea water for 5-7 h. Released algae sank through the mesh and into a sterile test tube. They were rinsed three more times by centrifugation in sea water and cultured in either PES/2 or in ASP8 (Provasoli 1968). Sterility tests were performed on the cultured algae as before. The

decrease in bacterial counts was evident on the STM agar plates where the number of bacterial colonies dropped from over 800 mL^{-1}1000 $amoebae^{-1}$ after the first rinse in PEG, to 0 ml^{-1}1000 $amoebae^{-1}$ after the fourth PEG rinse. The bacteria were diluted out after the fifth PES rinse (Fig. 2).

3) Migration on agar surfaces. The third method utilized the surface of 1.5% agar plates as crawling and releasing surfaces for the amoebae and as seeding grounds for the clean algae. In this method, the amoebae were starved for two weeks, treated in antibiotics for two days, rinsed three times in sterile sea water, centrifuged in 0.5% PEG two more times into a concentrated pellet, and placed on the surfaces of a solidified PES + 1.5% Difco Bacto-agar. The whole procedure was done axenically. The amoebae stretched and migrated on the agar while they went through multiple fission, thus releasing the algae (Fig. 3). The released algae were left to divide and develop colonies on the agar. The state of sterility of these colonies was determined through microscopic observations of the agar around the colony (after 5 days, under 400X). Antibiotic solution was added at times to the agar; however, growth rates of the algae were reduced on the antibiotic-enriched agar by a factor of three. The growth inhibition was reversible when the algae were transferred to PES/2 or to ASP8 media.

4) Disruption of killed amoebae to release clean algae. The fourth method was used only when none of the previous three procedures produced axenic algae. This was mostly due to the presence of motile eukaryotic contaminants which the starved

amoebae were not able to capture, or when antibiotic resistant contaminants were attached to the amoebal cell wall. In this method, the starved amoebae containg the algae were treated with antibiotics for two days and flooded for 10-30 sec with either a 0.5% perchloride solution in double distilled water, a 30% ethanol solution in DDW, DDW water alone, or a 10% Betadine solution in sea water. These chemical treatments killed the amoebae and the external contaminants but did not kill the algae protected inside the amoebal cytoplasmic vacuoles.

The dead but clean amoebae were collected axenically by centrifugation, rinsed twice, and teased apart manually using pulled glass pipets. For large numbers of amoebae, a small homogenizer (Biospec 1281) was used for 10 sec at speed 12. A probe sonicator was also useful to break the amoebae with minimal damage to the algae (15 sec, setting 4, on ice). The algae were collected by filtration through sterile nylon nets (20 um) and rinsed three times with sterile 0.5% PEG in sea water. Such algal preparations still contained some amoebal membranes even after excessive rinsing, but these did not inhibit the growth or cell division of the algae.

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Several fungi developed faster than the amoebae could phagocytose and digest them, and several species of ciliates and flagellates were too active to be captured.

RESULTS AND DISCUSSION

Out of the four methods described, method #1 was usually effective in producing axenic algae. Three isolates of Symbiodinium from the sea anemone Anthopleura were successfully cleansed using the first method. However, a fourth isolate of the same species contained a bacterium resistant to the antibiotics. For this isolate the agar surface (method #3) was used successfully. A fifth isolate of the same species contained a swift flagellate which had to be eliminated using a 0.5% perchloride solution or a 30% ethanol solution for 10 sec, as in method #4. Most species of Symbiodinium which were tested were resistant to the antibiotic mixture and were cleaned using methods #1 or #3. Symbiodinium #45, which turned pale in the antibiotic mixture, was cleaned through attachment to nylon netting (method #2) and Symbiodinium #344, which released sticky polysaccharides and collected particulate matter from the medium, was cleaned through the migration of the amoebae on agar, releasing algal cells in their trail (method #3). About 40% of the released algae developed axenic colonies on the agar surfaces. In order to eliminate a fast swimming spiral bacterium from the red unicellular alga Porphyridium, method #4 was used. It was also used for an isolate of Symbiodinium #8 and one of the

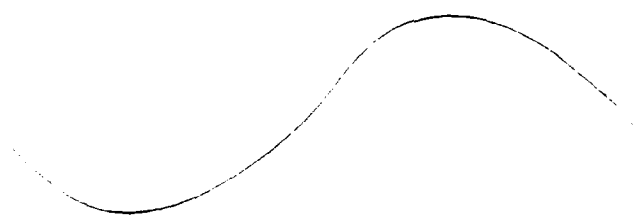
isolates of Symbiodinium anthopleura which contained swimming small flagellates resistant to the antobiotics and too motile to be engulfed by the amoebae.

With these four methods isolates of four species of the symbiotic dinoflagellate Symbiodinium (#8, 45, 61, and 344) and the red unicellular alga Porphoridium cinctum, emerged viable and axenic. These methods, and combinations and variations of them, may prove useful for cleaning other algae that are not digested, or digested slowly by various species of this and other protozoans.

TABLE 1. Antibiotics stock solution, 10X concentration. The solution was prepared in sea water (or fresh water) and diluted 1:10 in seawater for the amoebae, and in PES medium for the algae.

To 100 mL double distilled water (or sea water) add:

I		II	
Penicillin-G	1000mg	Erythromycin	6mg
Streptomycin	2000mg	Gentamycin	8mg
Kanamycin	1000mg	Polymixin-B	16mg
Neomycin	200mg	Tetracycline	12mg
Nystatin	15mg	Vancomycin	12mg



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REFERENCES

Fries, L. 1963. On the cultivation of axenic red algae. Physiol. Plant. 16: 695-708.

_____ 1977. Growth regulating effects of phenylacetic acid and p-hydroxyphenylacetic acid on Fucus spiralis in axenic culture. Phycologia 16:451-5.

Mooney, P.A. & Van Staden, J. 1985. In vitro plantlet formation and multiple shoot induction in Sargassum heterophyllum. S. Afr. J. Bot. 51: 41-4.

Polne-Fuller, M. 1987. A multinucleated marine amoeba which digests seaweeds. J. Protzool. 34: 159-65.

Polne-Fuller, M. & A. Gibor. 1987a. Calluses and callus-like growth in seaweeds: induction and culture. Hydrobiologia 151-152: 131-8.

_____ 1987b. Tissue culture of seaweeds. In: Bird, K. & Bensen, P.H. (Eds.) Seaweed Cultivation for Renewable Resources. Gas Research Inst., Chicago, pp. 219-40.

Provasoli, L. 1968. Media and prospects for cultivation of marine algae. In Watanabe, A. & Hattori, A. (Eds.) Culture and Collections of Algae. Japanese Society of Plant Physiologists, pp.63-75.

Rogerson, A., Polne-Fuller, M. & Gibor, A. 1989. A laboratory induced association between the marine amoeba Trichosphaerium Am-I-7 and the dinoflagellate Symbiodinium #8. Symbiosis 7: 229-41

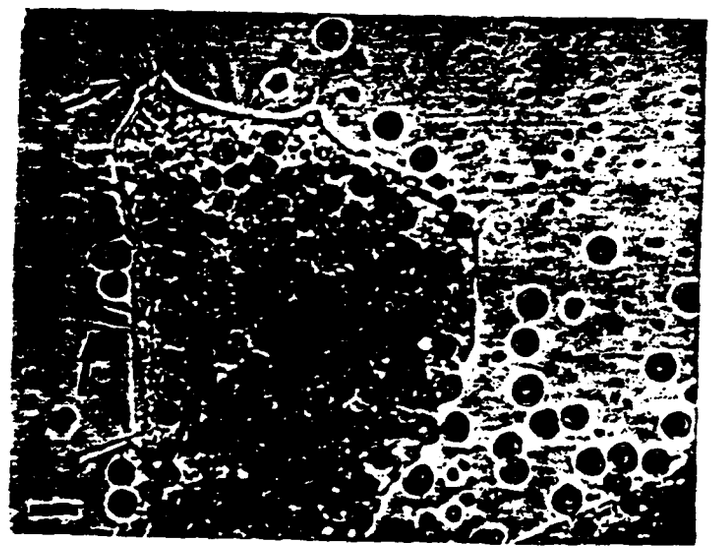
_____ 1990. Lectin binding sites in Marine amoebae. Arch. Protistenk. (In press).

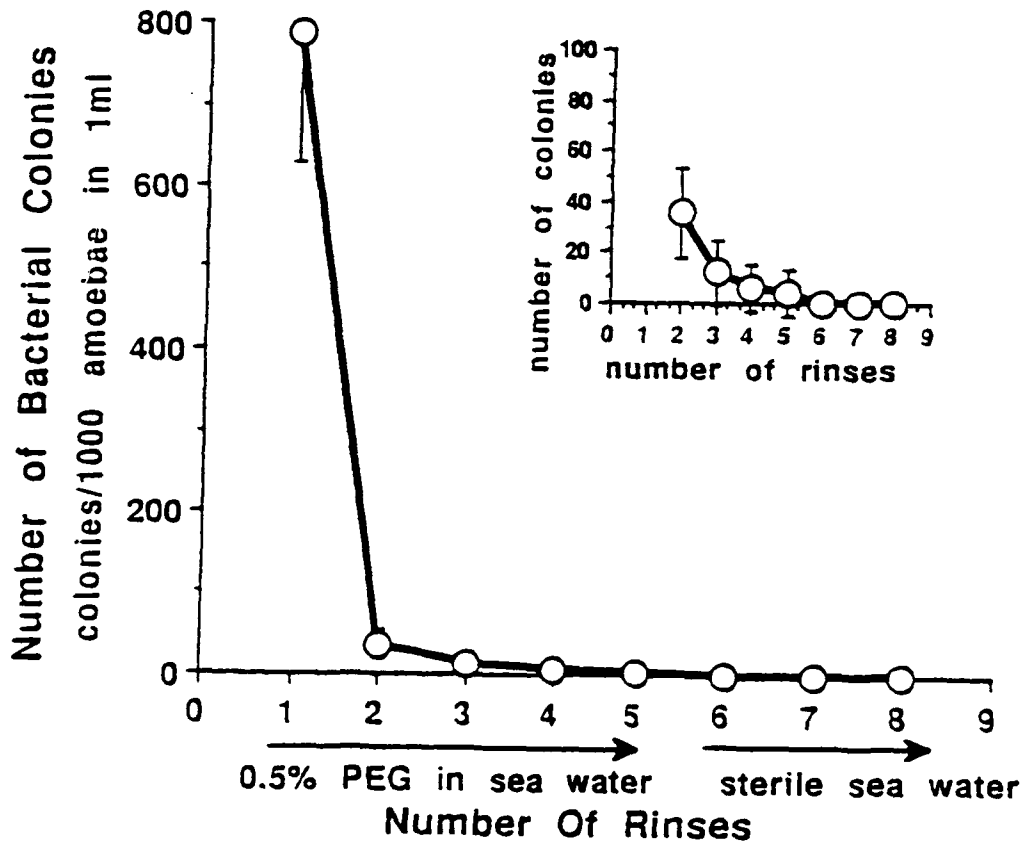
Figure Legends:

Fig. 1. A Photograph of Trichosphaerium Am-I-7 full of Symbiodinium #8. Note the amoebal filipodia (arrows), and the algae present inside and outside the amoeba. Bar = 15 um.

Fig. 2. Reduction in number of bacteria during rinses in polyethyleneglycole.
The smaller inserted graph presents an extended scale of the second through eighth rinses.

Fig. 3. Three amoebae migrating on agar surfaces, releasing algae on the agar surface enriched with antibiotics.
Bar = 20 um.




Number of Bacterial Colonies
colonies/1000 amoebae in 1ml
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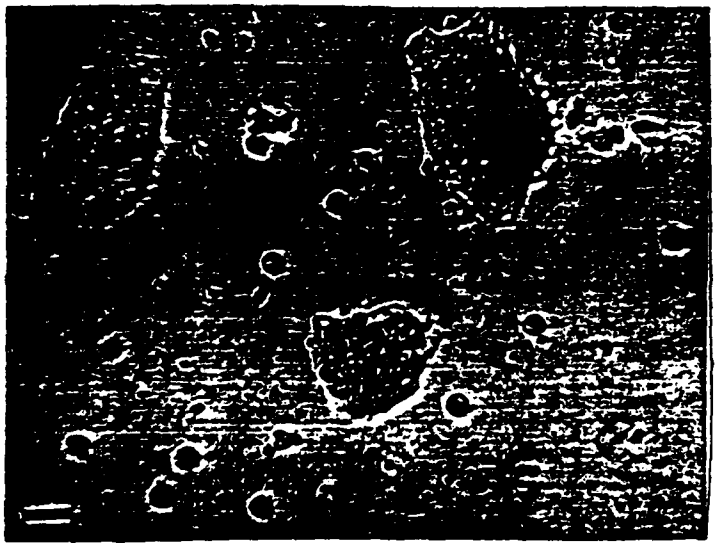
600
400
200
0
0 1 2 3 4 5 6 7 8 9
number of colonies
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9
number of rinses
0.5% PEG in sea water
sterile sea water
Number Of Rinses



significance, adaptations in ciliates and testacea, bioindicators and guide to the literature. *Progr. Protistol.*, 2. (in press)

10. Foissner, W. & Adam, H. 1983. Morphologie und Morphogenese des Bodenciliaten *Oxytricha granulifera* sp. n. (Ciliophora, Oxytrichidae). *Zool. Scr.*, 12: 1–11.

11. Hemberger, H. & Wilbert, N. 1982. Revision der Familie Keronidae Dujardin, 1840 (Ciliophora, Hypotrichida) mit einer Beschreibung der Morphogenese von *Kerona polyporum* Ehrenberg, 1835. *Arch. Protistenkd.*, 125: 261–270.

12. Jankowski, A. W. 1979. Revision of the order Hypotrichida Stein, 1859. Generic catalogue, phylogeny, taxonomy. *Proc. Acad. Sci. USSR*, 86: 48–85. (in Russian with English summary)

13. Jareño, M. A. & Tuffrau, M. 1979. Le positionnement des primordiums cinétosomiens et la morphogenèse sous kyste chez le cilié hypotriche *Onychodromus acuminatus*. *Protistologica*, 15: 597–605.

14. Jeffries, W. B. & Mellott, J. L. 1968. New observations on the anatomy of *Pleurotricha lanceolata*. *J. Protozool.*, 15: 741–747.

15. Jones, E. E. 1974. The protozoa of Mobile Bay, Alabama. *Univ. Alabama Monogr.*, 1: 1–113.

16. Kahl, A. 1932. Urtiere oder Protozoa I: Wimpertiere oder Ciliata (Infusoria) 3. Spirotricha. *Tierwelt Deutschlands*, 25: 399–650.

17. Lin, M. & Prescott, D. M. 1985. Electron microscope autoradiography of DNA synthesis in the replication band of two hypotrichous ciliates. *J. Protozool.*, 32: 144–149.

18. Martin, J., Fedriani, C. & Perez-Silva, J. 1983. Morphogenetic pattern in *Laurentiella acuminata* (Ciliophora, Hypotrichida): its significance for the comprehension of ontogeny and phylogeny of hypotrichous ciliates. *J. Protozool.*, 30: 519–529.

19. Neave, S. A. 1939–1975. *Nomenclator Zoologicus*. Vols. 1–7. Zoological Society, London, 4442 pp.

20. Schwoerbel, J. 1966. *Methoden der Hydrobiologie (Süßwasserbiologie)*. Franckh'sche Verlagshandlung, W. Keller & Co., Stuttgart, 207 pp.

21. Smith, L. F. 1914. A preliminary report on the infusoria of Kansas. *Kans. Univ. Sci. Bull.*, 9: 147–174.

22. Sokal, R. R. & Rohlf, F. J. 1981. *Biometry. The Principles and Practice of Statistics in Biological Research*. 2nd ed. Freeman, San Francisco.

23. Stein, F. 1859a. Characteristik neuer Infusorien-Gattungen. *Lotos (Praj.)*, 9: 2–5.

24. ——— 1859b. *Der Organismus der Infusionsthiere nach eigenen Forschungen in systematischer Reihenfolge bearbeitet. I. Abtheilung. Allgemeiner Theil und Naturgeschichte der hypotrichen Infusionsthiere*. Engelmann, Leipzig, 206 pp.

25. The Committee on Cultures, Society of Protozoologists. 1958. A catalogue of laboratory strains of free-living and parasitic protozoa (with sources from which they may be obtained and directions for their maintenance). *J. Protozool.*, 5: 1–38.

26. Wallengren, H. 1900. Zur Kenntnis der vergleichenden Morphologie der hypotrichen Infusorien. *Bih. Svensk. Vetens. K. Akad. Handl.*, 26: 1–31.

27. Wilbert, N. 1975. Eine verbesserte Technik der Protargolimprägnation für Ciliaten. *Mikrokosmos*, year 1975, pp. 171–179.

28. Wirnsberger, E., Foissner, W. & Adam, H. 1985. Cortical pattern in non-dividers, dividers and reorganizers of an Austrian population of *Paraurostyla weissei* (Ciliophora, Hypotrichida): a comparative morphological and biometrical study. *Zool. Scr.*, 14: 1–10.

29. ——— 1986. Biometric and morphogenetic comparison of the sibling species *Stylonychia mytilus* and *S. lemnae*, including a phylogenetic system for the oxytrichids (Ciliophora, Hypotrichida). *Arch. Protistenkd.*, 132: 167–185.

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A Multinucleated Marine Amoeba Which Digests Seaweeds[1]

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ABSTRACT. Marine amoebae were isolated during a search for organisms which degrade cell walls of seaweed. One of the isolates, a multinucleated amoeba (referred to here as Amoeba-I-7 or Am-I-7) was isolated from live tissues of the brown seaweed *Sargassum muticum*. It digested a variety of brown and red seaweeds including their walls and cuticles. Axenic clone cultures were isolated from cells that migrated on agar. Cultures were grown on agar or in liquid media. Seaweeds, seaweed wall extracts, and unicellular algae were tested as food sources.

AMOEBAE are a diverse group of protozoa with widely varied characteristics and habitats. Most amoebae in the marine environment are believed to be associated with detritus and to feed on bacteria, decaying plant and animal matter, or microscopic algae (3). Some amoebae were isolated from drifting specimens of the brown seaweed *Sargassum*. Since the same species were also isolated from water samples, it was assumed that the amoebae isolated from the seaweed fed on bacteria associated with the surfaces rather than on the plant tissue itself (5).

During our search for microorganisms capable of decomposing seaweed cell walls, several species of amoebae were isolated from intact seaweed tissues. These amoebae were clone-cultured axenically, and flourished on seaweed tissues free of debris or bacteria. Amoebae-I-7 (Am-I-7) was one of the isolates which was able to digest rapidly and completely a variety of brown and red seaweeds. This paper describes Am-I-7, its isolation and culture techniques, and the effectivity of various seaweeds as food sources.

MATERIALS AND METHODS

Isolation of Am-I-7. Young plants of *Sargassum muticum*, carrying amoebae, were collected in January 1984 from rocky shores at Alegria beach, Hollister Ranch, Santa Barbara County, California. Reproductive plants of *Sargassum* which were also infected were collected at the same site in April 1985. Moist

[1] The author would like to thank Mr. Kirk Apt and Mr. Su Xing for [illegible] of *Eucheuma* and *Chlamydomonas*, Mr. Robert Farris and Ms. [illegible] Richardson for EM consultations, Julie Young for technical [illegible], Dr. Aharon Gibor and Dr. Deborah Kaska for reading the manuscript, and Mr. Al Remmenga of the Hollister Ranch Association for clearing access to Alegria beach.

plants were transferred to the laboratory in a styrofoam container and stored at 5°C.

Sections of branches (1–2 cm long) were partially cleaned of surface microorganisms. Cleaning was done in chilled sterile seawater (5°C). The tissue was sonicated for 1 min (4 × 15 sec, with four sterile rinses between sonications) at setting 3 in an L&R sonicator (Ultrasonic 320, Kearny, NJ). The tissue was then soaked in 0.1% solution of bleach (5.25% sodium hypochlorite by weight) or in 30% ethanol for 2 min with gentle agitation. It was then rinsed well and incubated in antibiotics for three days. The antibiotic mixture contained: streptomycin sulfate (0.2%), penicillin-G (0.1%), neomycin (0.02%), nystatin (0.0015%), and kanamycin (0.1%). Sections of the antibiotic-treated tissue (2–5 mm) were placed on the surface of PES medium (12) solidified with 1.5% agar to which the same antibiotic mixture was added.

Clone cultures of Am-I-7 were isolated as cells migrated on the surface of the agar. To assure absence of contaminating bacteria, the isolates were cultured initially on sterility-test medium which contained 1.5% agar in PES medium (12) enriched by 0.8% nutrient broth, 0.5% yeast extract, and 0.2% sucrose.

Special precautions had to be taken during isolation and transfer. The cells were delicate and ruptured easily if the agar surface beneath them was stretched. Exposure to desiccation was also detrimental. Transfer from agar surfaces was done either by lifting a cell with the section of the agar or by the use of a hair loop to which the cell attached. Transfer from liquid cultures was done by gentle pipetting. The cells were quickly removed from the pipette before they attached to it.

Growth measurements. For feeding experiments, 20 large amoeba cells (60–80 μm) were placed in 4-cm diameter plastic dishes containing 250 mg live, sterile algal tissue in 20 ml of seawater. The bottoms of the dishes were marked with a 5-mm grid. The total number of cells were counted daily for two weeks. When the number of cells became too large to count, 10 sample areas on the bottom were counted.

An American Optics dissecting microscope was used for observations at low magnification (30×). An inverted Nikon microscope was used for observations of cell morphology and movement at higher magnification (100–1000×). A Reichart phase/epifluorescent microscope was used for fluorescence work.

Food sources. 1. Agar—Difco Bacto-agar or Sigma Agarose were prepared in seawater and used at concentrations ranging between 0.1 and 1.5%. Fifteen milliliters of medium were poured into 4-cm diameter plastic petri dishes. 2. Carrageenan—Carrageenan (Irish Moss, Type I, Sigma) autoclaved in seawater was used. A range of concentrations from 0.5 to 2.5% was prepared and poured as above. 3. Algin—Kelmar Algin (KR-2329-69, Kelco Company) was autoclaved in seawater. Concentrations of 0.5–2.5% were prepared as above. 4. Seaweeds—Live, axenic, seaweed tissues to be used for feeding were prepared as previously described (9–11). The cleaned tissues were placed in culture dishes with seawater and the amoebae transferred on top of the tissue.

Autoclaved seaweeds were used for feeding large scale cultures. For this, the seaweeds were cleaned of visible epiphytes and chopped in a blender for 2 min. The chopped tissues were rinsed in seawater and autoclaved for 20 min (500 g wet tissue/liter). For long storage, air-dried seaweeds were ground in a coarse mill grinder (Arthur Thomas Corp., Philadelphia) and stored dry. Before feeding, the coarse powder was autoclaved in seawater (5 g/100 ml), let soak for a day, rinsed twice, and resterilized in seawater. 5. Unicellular algae—Unicellular algae were harvested by centrifugation and added to the amoebae as a suspension. Flagellated algae were frozen before feeding to stop motility. Amoeba-I-7 was also cultured monoxenically with the marine unicellular algae. Combined cultures require no feeding and were most convenient for observations of behavior, growth, and cell division.

Nuclear staining. Live Am-I-7 cells were placed on a slide in a 0.01 μg/ml solution of 4'-6'-diamidino-2-phenylindole (DAPI stain, Sigma) in seawater. Alternatively, the cells were fixed in acetic-acid : ethanol (1:3), then rinsed in fresh water before staining with DAPI. Acridine Orange (0.01% in seawater) was also effective for staining nucleic acids of Am-I-7.

OBSERVATIONS AND DISCUSSION

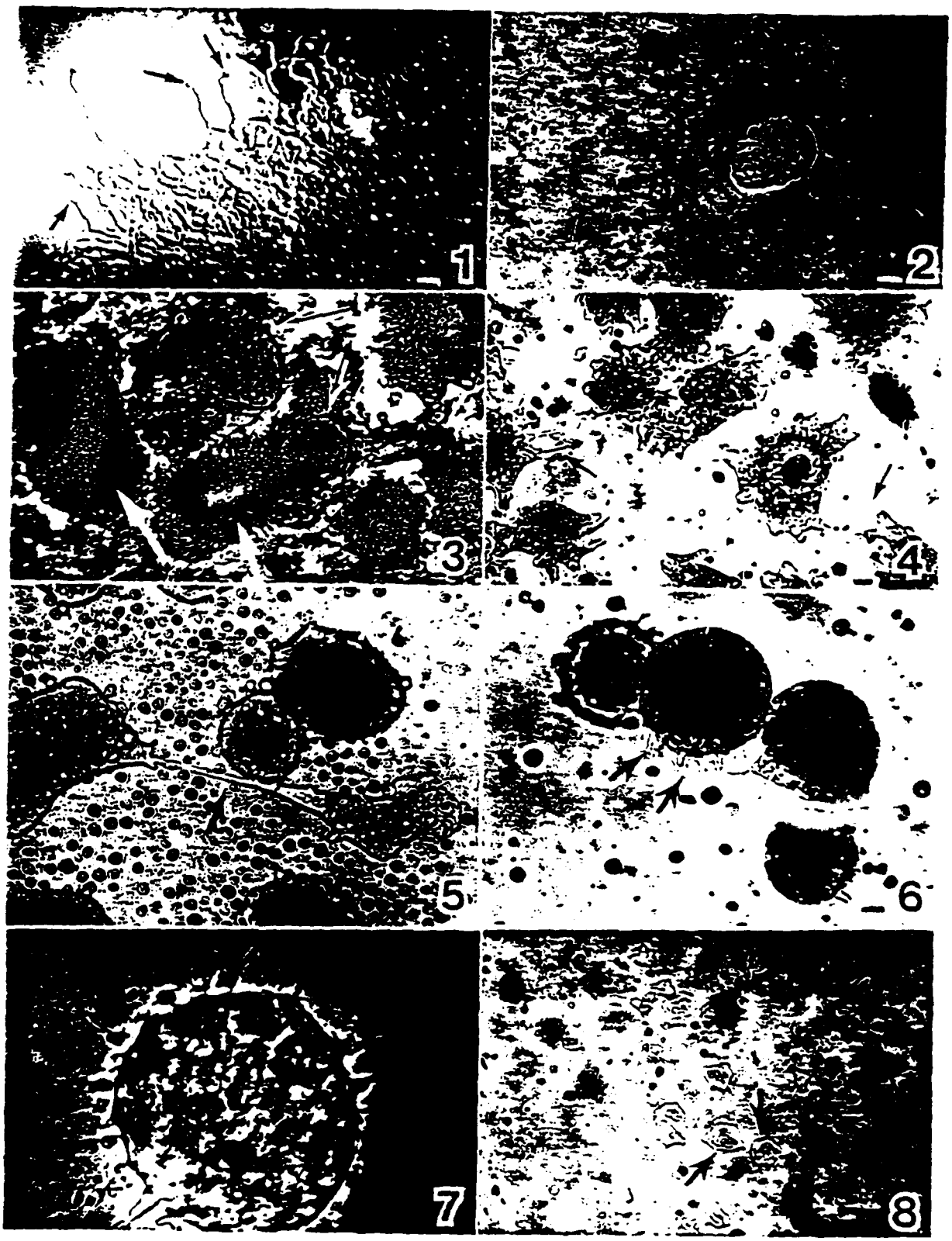
Isolation of amoeba cells. Although many different seaweed species were collected year round and tested for the presence of amoebae, Am-I-7 was isolated only twice and only from *Sargassum muticum*. In January 1984 it was present in small numbers on stipes of young *Sargassum* plants. In April 1985 it was found in large numbers on reproductive plants of this seaweed.

In culture, blooms of competing bacteria were detrimental to the amoebae and had to be eliminated. Partial cleaning of seaweeds with mild bleach or alcohol eliminated most of the microbial population on the seaweed surface. The few organisms which survived on the seaweed may have been located within the tissue, escaping the surface treatment, and Am-I-7 was one of them. Individual amoeba cells migrated away from the seaweed, on and through the agar, and created visible tunnels as they digested the gel in their path (Figs. 1–3).

Cell morphology. AM-I-7 resembles in many respects *Amoeba tentaculata* (7), or *Pontifex maximus* (13), or the smooth form, the gamont of *Trichosphaerium platyxyrum* (1, 2, 6, 7, 15, 16). There has been an earlier suggestion that all three are the same organism (8).

Alternation of generations in *Trichosphaerium* was first suggested by Schaudinn (14). A smooth form and a form covered with spicules were later identified as the gamont and the schizont stages in *Trichosphaerium*'s life history (4). Although the question of alternation of generations was never confirmed, alternation of morphology was reported by Angell (1, 2) who found smooth forms, suspected gamonts, in cultures of the "fuzzy" or

Figs. 1–8. Photomicrographs of living cells of Amoeba-I-7. 1. Cells migrating in their tunnels in agar. A cell is visible at the end of each tunnel (arrows). Bar = 200 μm. 2. An amoeba at the head of its tunnel. Fecal pellets can be seen as white particles in the tunnel. Note the vacuoles on the cell's surface. Bar = 20 μm. 3. Two-week-old culture on agar. Amoebae (black arrows) digesting large hole in the agar (white arrows). Note the granular deposits of fecal pellets in the tunnels and craters. Bar = 50 μm. 4. Cells in log-phase culture. These cells were fed on fresh branches of *Sargassum muticum*. The distinct dark center of the middle cell contained a recently engulfed section of seaweed. Clear lobopodia can be seen and thin filopodia extended through cones formed by the test (arrows). Bar = 50 μm. 5. Amoebae fed on *Chlamydomonas reinhardtii*. Arrow points at a binary fission. The thin cytoplasm broke as the daughter cells moved away. Cells of *Chlamydomonas* are in the background. Bar = 30 μm. 6. Well-fed amoebae free of the substrate. Dactylopodia (arrows) were used for movement. The small cells in the background are *Chlamydomonas*. Bar = 20 μm. 7. Echinate-truncate protrusions on detached cell. Note the fine dactylopodia extended from the cytoplasmic cones. This cell fed on dinoflagellates. Bar = 15 μm. 8. Progeny of an amoeba 24 h after a multiple fission. The progeny were 20 μm in diameter, flat, and migrating. Note the dense area in the cytoplasm (arrows) and the "fan-like" clear cytoplasm at the front of the cell. Bar = 15 μm.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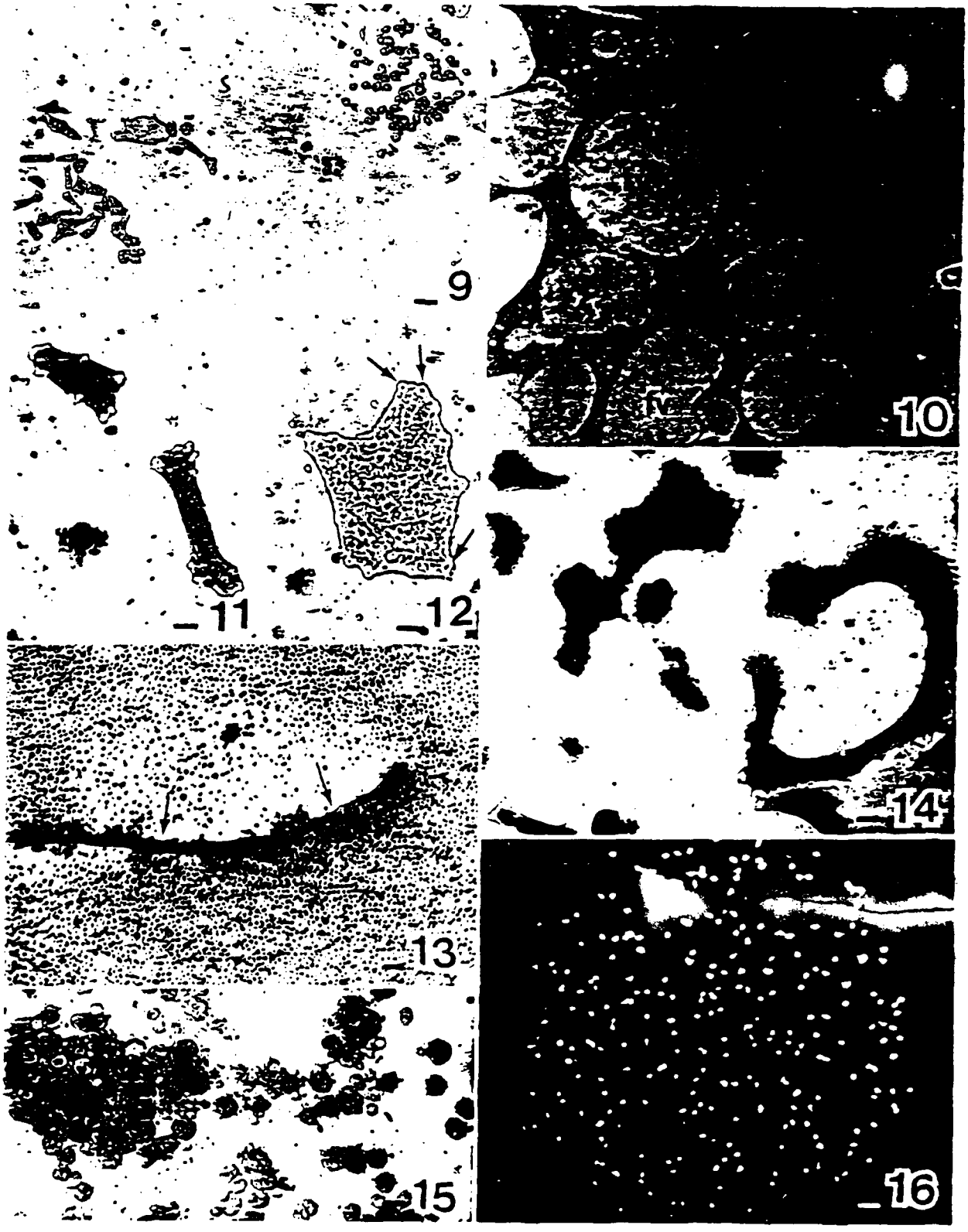
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schizont form. Griffin & Spoon also reported "fuzzy" forms of *Trichosphaerium* which were obtained by environmental manipulation of cloned cultures of the "smooth" form. The nature of the manipulations was not described (abstract, Intern. Cong. Protozool., 1977). Although a wide range of culture conditions and food sources were tested in axenic clonal cultures during two years of the present study, a "fuzzy" or schizont morphotype was never observed.

Under optimal culture conditions (22°C, 31 ppt salinity, and ample food) adult cells were amoebcid, actively moving plano-convex bodies, 40–120 μm in diameter; however, the size of the cells ranged from 10–>1000 μm, depending on their age and recent feeding history. Active cells were tightly attached to a substrate, feeding constantly and full of particles which determined their coloration (Figs. 4–7).

The "youngest" cells (see multiple fission below) were small (10–20 μm), flat, with stretched, hyaline, fan-like cytoplasm (Fig. 8). In time (3–7 days) and with abundant feeding, these grew into the large plano-convex flat cells (40–120 μm), which were full of dense cytoplasm loaded with food vacuoles and storage granules. Clear and cylindrical, echinate-truncate, cytoplasmic protrusions (5–15 μm long, 4–6 μm wide) appeared and disappeared on the clear cytoplasm covering the cell. Their truncated fronts were blunt and ended with a small crater (3–5 μm diameter), suggesting the presence of a test (Figs. 4, 7, 10). Fine dactylopodia extended from the echinate-truncate cytoplasmic protrusions (Figs. 4, 7) and waved slowly back and forth before retracting. Many other amoeboid cell configurations also existed. Among them were smooth, pointed, round, or elongated cells (Figs. 2–9, 11–15).

Large groups of detached, greenish-beige, spherical cells, were common in cultures that were starved for four to eight weeks (Fig. 15). Feeding of starved cultures resulted in very long and thin cells (1000 μm × 30 μm), tightly attached to the substrate and eagerly "mowing" the surface for food (Fig. 13). Elongated forms lasted from a few hours to a day; then the cells resumed the more common 50–100 μm diameter, plano-convex shape.

Detached, pigmented, spherical cells (40–200 μm) with active podia were common 1–2 days after feeding (Fig. 6). The well-fed cells were settled on the bottom and slowly wandered along, transferring their center of gravity from one set of dactylopodia to another as they were advancing. Lobopodia were short or missing in these cells and were not used for locomotion; however, the dactylopodia were extended from clear lobopodium-like areas, which may have given them physical support. The well-fed, suspended morphology lasted for 3–7 days, after which the cells re-attached and started feeding and dividing again.

The color of the amoebae and the apparent consistency of the cytoplasm depended on the food source. Cells which were fed on unicellular green algae were dark green and granular. Each of the numerous food vacuoles contained from one to four well-packed algal cells (Figs. 5, 6). Amoebae which were fed on dinoflagellates or brown seaweeds were golden to dark brown with less granulation, depending on the type of seaweed they engulfed (Fig. 7). Those that fed on agarophytic red seaweeds or on agar, carrageenan, fucan, or alginate had finely granulated cytoplasm and a light beige color (Fig. 2). When fed on the red unicellular alga, *Porphyridium*, the amoebae were bright red and granulated, and when sugar solutions were the provided carbon source, the amoebae were silvery-greenish and nearly clear.

Dactylopodia and lobopodia. Fine needle-like dactylopodia (2–4 μm wide, 5–50 μm long) were extended from the echinate-truncate cytoplasmic protrusions on the cell surface or cell test (Figs. 4, 6, 7). Dactylopodia remained extended for 0.5–10 min and waved slowly and continuously. The cytoplasmic protrusions retracted with the dactylopodia or remained extended, ready to accommodate a new set. Dactylopodia formed in all directions with larger numbers in the direction of movement. They formed in groups of 1–3 (rarely 4) per protrusion (Figs. 4, 7). Most were a single cytoplasmic filament, few branched once, forming a fine fork. Dactylopodia did not seem to take an active role in movement of flat attached cells. They were used, however, for motion in detached cells which were supported by dactylopodia and moved by transferring their weight on them in a bumpy motion.

Lobopodia were most common on flat, attached cells, where they were used for movement (Figs. 4, 8). In small cells, a wide lobopodium of clear cytoplasm was extended in a round front while the mass of granular cytoplasm slowly streamed into it. Observations of attached moving cells on glass or agar beads indicated that the test and cell membrane were rolling while the cytoplasm was streaming.

Dactylopodia were thin and extended from the cytoplasmic echinate-truncate protrusions. Lobopodia were round and were not observed in association with these cone-like protrusions; however, these two types of podia appeared to be somehow interchangeable. On several occasions the typically 2-μm-wide, 15–20-μm-long dactylopodia gradually changed their morphology. Within a few seconds they were transformed into a 30–40-μm-wide lobopodium, which was then either retracted, widened further, or reshaped as a thin dactylopodium. The echinate-truncate cytoplasmic protrusions moved with the surface membrane around the cell, changing their positions relative to each other and to the cell edges. It is not clear whether these were permanent structures or a transient formation of cell cytoskeleton. Light and electron microscope observations did not reveal a permanent structure in the cell membrane or the cell test which corresponded to these cytoplasmic protrusions.

Cell division. Two types of cell divisions occurred: binary fission and multiple fission. Binary fission occurred in well-fed cultures. Cells larger than 60 μm in diameter divided, forming two daughter cells not always identical in size nor in number of nuclei. In preparation for division, opposite poles of one cell moved in opposite directions and a thin cytoplasmic bridge

Figs. 9–16. Changes in appearance of Amoeba-I-7 with changes in physiological state. 9. Progeny of multiple fissions (two cells) before active migration started (7 h after division started). The top right cell, originally 110 μm, produced 72 progeny. The left cell, originally 140 μm was not yet finished dividing. Its 24 visible progeny continued to divide and reached their final size (20 μm) 2 h later. Bar = 20 μm. 10. Transmission electron micrograph of a log-phase cell. The clear food vacuoles (fv) are full of agar. A test is present at the cell surface (arrows; × 30,000). 11. Three amoeba cells with typical round to dumb-bell shapes. Cells tended to assume the dumb-bell shape before binary fissions. Bar = 30 μm. 12. Flat cell after five weeks of starvation. Bar = 50 μm. 13. An Am-I-7 cell exposed to *Dunaliella* after being starved for six weeks. Each amoeba cell formed a long rod to maximize scraping ability. Note the cleared area the cell left behind. Both arrows point at one cell and its direction of movement. Bar = 40 μm. 14. Extremely large cells were formed by several cell fusions in well-fed cultures. Bar = 80 μm. 15. Groups of cells starved for six weeks floating in the medium. The small particles are fecal pellets. Bar = 100 μm. 16. A DAPI-stained cell, 180 μm in diameter, with brightly fluorescing nuclei distributed in the cytoplasm between the round food vacuoles. About 267 nuclei can be counted. Bar = 12 μm.

TABLE 1. *Growth rates of Am-I-7 on different algae.*

Food source	Divisions/week*	Live-tissue boiled	Quality
Green seaweeds			
Enteromorpha intestinalis	0	1–2	Poor food source
Ulva angusta	0–0.5	1–2	Poor
Cladophora columbiana	0–0.6	2–3	Poor
Unicellular green algae			
Chlamydomonas reinhardii	9–11	—†	Very good
Dunaliella tertiolecta	7–10	—	Very good
Platymonas subcordiformis	8–9	—	Very good
Carteria pallida	8–9	—	Very good
Nanochloris sp.	9–10	—	Very good
Acetabularia mediterranea	0–0.5	—	Poor
Brown seaweeds			
Macrocystis pyrifera gametophytes	10–11	—	Very good
Macrocystis pyrifera sporophytes	8–10	9–10	Very good
Laminaria farlowii sporophytes	8–9	8–10	Very good
Sargassum muticum	5–7	6–7	Good
Sargassum filipendula	7–8	7–8	Good
Sargassum natans	7–8	7–8	Good
Sargassum fluitans	7–8	7–8	Good
Sargassum pteropleuron	7–8	7–8	Good
Sargassum hystrix	6–8	6–9	Good
Cystoseira osmundacea	5–8	5–8	Good
Zonaria farlowii	5–7	5–8	Good
Red seaweeds			
Porphyra perforata conchocelis	3–5	—	Poor
Porphyra perforata blades	6–7	7–8	Good
Gracilaria sjoestedtii	7–9	7–10	Very good
Gracilaria andersonii	8–10	8–10	Very good
Gelidium robustum	7–9	7–10	Very good
Prionitis lanceolata	8–9	8–10	Very good
Eucheuma alvarezii	8–9	8–10	Very good
Gigartina exasperata	8–9	7–10	Very good
Gigartina papillata	8–9	8–10	Very good
Commercial seaweed wall extracts			
Algin (Kelco)	—	6–7	Good
Agar (Difco)	—	7–9	Very good
Carrageenan (Sigma)	—	7–9	Very good

* Growth experiments were done with large cells (60–80 μm) dividing by binary fission.
† Not tried.

remained fast before the cells separated (Fig. 5). Minimum cell size and the presence of sufficient food supply were two necessary requirements for binary fission.

Extremely large cells (500–1000 μm) went through a chain of binary fissions. As the oversized cells were dividing and before the first division was completed, a second, third, or fourth binary fission was initiated. Such large cells produced a chain of as many as 11 daughter cells during a sequence that lasted 10 min.

Starved cells retained their size for eight weeks and then shrank slowly, becoming clear and barely recognizable. Feeding of starved cells restored their normal range of sizes in 3–10 days.

Multiple fission was a second type of cell division. It was induced by exposing large cells to fresh PES or seawater medium. Induction of multiple fission was recognizable 3–5 h after the medium was replaced. Depending on culture conditions (pH, salinity, temperature) and the physiological state of the cells, it lasted 5–48 h. Each cell (60–>1200 μm) divided into 10–>261 progeny, depending on the original size of the cell. During multiple fissions the cells released the contents of their food vacuoles. The young cells remained nearly motionless for 5–10 h after division (Fig. 9). They were 7–10 μm in diameter, slightly wrinkled, and whitish-beige. Twenty hours later, the cells were moving actively. They were clear and fan-like and stretched to 15–20 μm, attached to the substrate (Fig. 8). Their dense cytoplasm accumulated in one side of the cell, and a clear area with dense cytoplasmic ridges was fanned in the direction of movement. Young small cells started feeding as soon as they started moving. They could also survive eight weeks of starvation.

The induction of multiple fission was most reliable and was used for fast production of large numbers of cells. Multiple fissions resulted in 15–80-fold increase in progeny per division as compared to binary fission.

Nuclei. Nuclei that were stained by DAPI fluoresced bright blue. As many as 450 of them were counted in large cells (200 μm). The number of nuclei was proportional to the cell size. After multiple fission the nuclei were distributed between the progeny, which contained 1–5 nuclei per cell. The nuclei were about 2–3 μm in diameter and evenly distributed in the cytoplasm between the food vacuoles (Fig. 16).

Movement. In liquid cultures, while attached to the culture dish, Am-I-7 moved at about 10 μm/min. Continuous observations revealed that the cells spent about 50% of the time extending and retracting lobopodia and dactylopodia in what seemed to be a random fashion, without changing their position. Over several hours the cells moved in a winding path, engulfing the food particles in their way. They moved by extending wide clear lobopodia, which attached to and wrapped around solid surfaces.

When directional movement occurred, dense cytoplasm first streamed into a clear lobopodium, and the whole cell gently rolled following the direction of movement. The movement of attached cells was gradual with no sudden changes; however, sudden motions were common when cells encountered an unexpected disturbance, bristles of a brush or a hair loop. In reaction, the cells quickly released their contact with the substrate and pulled their cytoplasm away from the disturbance. Less than a minute later the cells were actively testing the intruder, and if no further motion of the intruder occurred, it was used as substrate.

Detached cells in stirred liquid medium were carried by the medium without control over their movement although their clear protrusions and dactylopodia were extended ready to attach. In still liquid the dactylopodia of detached cells supported the cells, keeping them above the substrate. The cells moved slowly on the tips of these fine cytoplasmic extensions.

On agar, each moving cell created a tunnel which marked the cell's path in the agar. It is not yet known whether the cells released short-lived digestive enzymes into the path to create the tunnels, or whether membrane-bound enzymes were involved. It was clear though that the agar surrounding the tunnels was not dissolved, and that the content of the tunnels was consumed. The tunnels contained colorless, shiny particles (Figs. 2, 3) resembling the fecal pellets observed in cultures fed on agarophytic seaweeds. The pellets did not contain proteins and melted at 60°C, suggesting agar components which were not digested by the amoebae and were released in small bundles.

A cell was always found at the head of its path (Figs. 1–3). Cell movement on agar initially concentrated at the surface of the gel. Well established cultures had tunnels distributed

throughout the agar. They also contained areas where cells divided at tunnel fronts without migrating. Such cell accumulations resulted in large holes in the partially digested agar (Fig. 3). Besides the amoebae (Fig. 3, black arrows), the holes were full of the shiny released contents of food vacuoles or fecal pellets (Fig. 3, white arrows).

Cytoplasmic exchange. Movement of cytoplasm between cells was observed in dense cultures. When contact between cells was common, cells (>60 μm) formed cytoplasmic bridges, 30–50 μm wide. Portions of the cytogel (20–30% of the cell volume) were observed being exchanged simultaneously between the cells. The process lasted 5–60 min. Then cell membranes reformed, replacing the bridge, and the cells separated and moved away from each other. During such exchanges the two cells retained their individual morphology although their cytoplasm was bridged.

Food and feeding. Amoeba-I-7 engulfed food particles which it encountered on its path. It was also readily able to "cut" pieces of live solid seaweed tissues and of various hard gels made of seaweed wall extract. The cells crawled on many available substrates, but they clearly recognized edible from nonedible particles. They did not engulf their fecal pellets even when starved. They attached to but did not engulf glass beads unless they were coated with agar, and they selectively collected algal species which were more digestible.

A variety of seaweed tissues, live and boiled, as well as plain agar, carrageenan, algin, and fucans supported good growth of Am-I-7. Live green seaweeds (*Enteromorpha, Ulva, Cladophora, Chara, Acetabularia*) were the least edible. When supplied with green seaweeds, the amoebae accumulated on the surfaces of the plants and collected particles and small amounts of released cytoplasm from broken cells, but growth did not occur. Boiled green seaweeds were more digestible and supported marginal growth at rates of 1–2 cell divisions per week. In comparison, brown and red seaweeds supported good growth of 7–10 divisions per week (Table I). Among the brown seaweeds, the Laminariales supported the best growth. *Macrocystis* gametophytes were a preferred food source. *Macrocystis* sporophytes were also a preferred food source although they required a longer period of digestion than the gametophytes. *Sargassum, Cystoseira,* and *Zonaria* supported lower initial growth, but once the cultures were established, division rates increased.

Among the red seaweeds the agarophytes and carragenophytes were an excellent food source for Am-I-7. *Porphyra* cuticle was a difficult surface for the amoebae to penetrate although a week after inoculation the amoebae were multiplying well in between the cuticle sheets of this plant. Boiled *Porphyra* cuticles were more accessible but not totally digestible.

Besides being an interesting biological system Am-I-7 is also an easily cultured source for degrading enzymes for seaweed cell wall dissociation. The lack of such enzymes has been a major barrier in isolation of single cells and protoplasts from many seaweeds. Seaweed protoplasts are desirable for studies in basic biology of algal cells and in the process of transforming the weeds of the oceans into cultivated crop plants.

LITERATURE CITED

1. Angell, R. W. 1975. Structure of *Trichosphaerium microrum* sp. n. *J. Protozool.*, 22: 18–22.
2. ——— 1976. Observations on *Trichosphaerium platyxyrum* sp. n. *J. Protozool.*, 23: 357–364.
3. Bovee, E. C. & Sawyer, T. 1979. Marine flora and fauna of the Northeastern United States. Protozoa: Sarcodina: Amoebae. *NOAA Tech. Report NMFS circular 419. U.S. Dept. of Commerce, NOAA, and National Marine Fisheries Service.* 56 pp.
4. Calkins, G. N. 1933. *Biology of the Protozoa.* 2nd ed. Lea & Febiger, Philadelphia. 457 pp.
5. Davis, P. G., Caron, D. A. & Sieburth, M. 1978. Oceanic amoebae from the North Atlantic: culture, distribution, and taxonomy. *Trans. Amer. Microsc. Soc.*, 97: 73–88.
6. Grell, K. G. 1968. *Protozoologie.* 2nd ed. Springer-Verlag, Berlin-Heidelberg-New York. 385 pp.
7. Gruber, A. 1881. Beitrage zur Kenntniss der Amoeben. *Z. Wiss. Zool.* 36: 459, pl. XXX.
8. Page, F. C. 1983. *Marine Gymnamoebae.* Cambridge Inst. of Terrestrial Ecology. pp. 45–47.
9. Polne-Fuller, M., Biniaminov, M. & Gibor, A. 1984. Vegetative propagation of *Porphyra perforata. Hydrobiologia.* 116/117: 308–313.
10. Polne-Fuller, M. & Gibor, A. 1984. Developmental studies of *Porphyra* species. I. Cell differentiation and protoplast regeneration in *Porphyra perforata. J. Phycol.*, 20: 609–616.
11. ——— 1987. Tissue culture of seaweeds. *in* Bird, K. & Benson, P. H., eds., *Seaweed Cultivation for Renewable Resources.* Gas Research Inst., Chicago. (in press)
12. Provasoli, L. 1968. Media and prospects for cultivation of marine algae. *in* Watanabe, A. & Hattori, A., eds., *Cultures and Collections of Algae.* Proc. U.S.-Japan Conf. Hakone. Sept. 1966. Jpn. Soc. Plant Physiol., Tokyo. pp. 63–75.
13. Schaeffer, A. A. 1926. The taxonomy of the amebas with description of 39 new marine and fresh water species. *Carnegie Inst. Wash. Publ.*, #345.
14. Schaudinn, F. 1899. Untersuchungen uber den Generationswechsel von *Trichosphaerium sieboldi* Schn. *Abh. Preuss. Akad. Wiss. Phys.-Math. Berlin.* (Suppl.), pp. 1–93.
15. Sheehan, R. & Banner, F. T. 1973. *Trichosphaerium*, an extraordinary testate rhizopod from coastal waters. *Estuarine Coastal Mar Sci.*, 1: 245–160.
16. Schneider, A. 1878. Beitrage zur Kenntniss der Protozoen. *Z. Wiss. Zool.* 30(Suppl.): 446, pl. XXI.

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Digestion of seaweeds by the marine amoeba *Trichosphaerium*

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Abstract

A crude enzyme preparation from the marine amoeba *Trichosphaerium* was used to produce protoplasts from *Sargassum muticum*, *Macrocystis pyrifera*, *Porphyra perforata*, and other red and brown marcroalgae. Cortical and medullary protoplasts of *Sargassum*, which were impossible to obtain using mixtures of previously available enzymes, have now been prepared. Intact inner cortical and medullary protoplasts of *Macrocystis*, which were not observed in past isolations, were obtained. Improved protoplast yields of as much as 500 fold resulted from feeding the amoebae on specific seaweeds. Cuticles of live *Sargassum* and *Macrocystis* were digested easily by the amoebae. However, cuticles of autoclaved *Macrocystis* and those of *Porphyra* (fresh or autoclaved) were eaten last. In spite of the absence of identifiable extracellular enzymatic activity in the medium, the amoebae were able to 'cut' and consume live fronds and blocks of gelled agars, carrageenans, and alginates.

Introduction

Seaweed grazers and pathogens have been used extensively as sources for seaweed cell wall degrading enzymes (Cheney *et al.*, 1984; Liu *et al.*, 1984; Polne-Fuller & Gibor, 1984; Fujita & Migita, 1985). These available enzymes were limited in their ability to degrade different algae and different tissue types. Therefore, we have been searching for new sources of more efficient enzymes with a wider range of substrates.

This paper describes the use of amoebal enzymes for degradation of seaweed tissues and for protoplast isolation.

Materials and methods

The marine amoeba *Trichosphaerium Am-I-7* was isolated from tissues of *Sargassum muticum* (Yendo) Fensholt collected locally at Alegrea Beach, Hollister Ranch, Santa Barbara, California (Polne-Fuller, 1987a). Three major experimental plants were used: *Macrocystis pyrifera* (L.) C. Agardh was collected locally at the University of California, Santa Barbara campus point, *Sargassum muticum* at Santa Barbara Harbor, and *Porphyra perforata* J. Agardh off rocks at UCSB campus point. Other species [*Egregia menziesii* (Turner) Areschoug, *Gelidium robustum* (Gardner) Hollenberg et Abbott, *Gigartina exasperata* Harvey et Bailey, *Gracilaria andersonii* (Grunow) Kylin, and *Prionitis lanceolata* (Harvey) Harvey] were all collected at Coal Oil Point, Goleta, and at Alegrea Beach, Hollister Ranch, California. *Eucheuma alverzii* Doty and *Eucheuma striatum* Schmitz were collected in Hawaii, and *Eucheuma uncinatum* Setchell et Gardner was originally collected in Baja California and then

cultured in the laboratory. Actively growing seaweeds were used. Splitting frond meristems and stipe tissues ~30 cm below the frond meristem of *Macrocystis* were selected. Young secondary branches and developing holdfast shoots of *Sargassum*, and young actively growing *Porphyra* blades were used. The tissues were cleaned and prepared for protoplast isolation as previously described (Polne-Fuller, 1987b; Fisher & Gibor, 1987). The cleaned tissues were either immediately processed or placed in full strength PES medium (Provasoli, 1968) at 60 μmol m^{-2} sec^{-1}, 18 °C, 14 : 8 L : D cycle, for three to five days prior to being chopped for protoplast isolation.

The amoebae were cloned and cultured on sterile seaweeds (axenic, live-cultured algae or autoclaved tissues collected from nature). Digestive enzymes were induced by feeding the amoebae on a specific seaweed tissue for one to three weeks. In experiments to induce specific digestive enzymes, the food source was switched every three weeks, and amoebal extracts were tested on the various seaweeds that were used as feed. Enzyme activity was determined by protoplast yields (# protoplasts per g wet tissue) after two, four, six and ten hours of digestion. The enzyme source was crude extract of 10^7 amoebae mL^{-1} phosphate buffer (pH 6.0). The extracts were prepared by one of two methods:

1. Amoebae (10^7 mL^{-1}) were sonicated for 15 seconds at 200 Watts in phosphate buffer containing 0.6 M mannitol. The extract was centrifuged (15000 rpm), the supernatant collected, its pH adjusted to 6.0, and the solution was filter-sterilized (0.22 μm).

2. Amoebae (10^7 mL^{-1}) were hand-ground in a glass-on-glass homogenizer in sea water containing 0.6 M sorbitol. The extracts were cleared by centrifugation (15000 rpm), adjusted to pH 6.0, and filter-sterilized (0.22 μm).

Axenic seaweed tissues were finely chopped (< 1 mm) and the enzyme solution added (about 1 g wet wt mL^{-1}). Large volume treatments (10 mL enzyme solution + 5–10 g wet tissue) were placed in petri dishes (6 cm diameter) and agitated on an orbital shaker (30 rpm) at room temperature. Rotation on a tissue culture wheel was effective as well. Small volumes (500 mg in 0.5 mL enzyme solution) were incubated in depression slides covered by a thin layer of mineral oil, without agitation. Incubations were done at room temperature under low light (7–17 μmol $m^{-2}sec^{-1}$).

Results and discussion

Spontaneous cell wall degradation, i.e. presence of protoplasts, was not detected in actively growing amoeba cultures even after two months of feeding. Filtered medium in which amoebae were actively feeding did not soften tissues or release protoplasts, indicating lack of enzyme activity in the medium, whereas crude extracts of disrupted amoebae were enzymatically active and released healthy protoplasts. These crude enzyme preparations were not toxic to the cells, eliminating the need for enzyme fractionation or purification.

Protoplasts from certain tissue types of *Macrocystis*, *Sargassum*, and *Porphyra* were prepared previously by treatments with mixtures of commercial and freshly prepared enzymes (Fisher & Gibor, 1987; Kloareg *et al.*, 1989; Polne-Fuller & Gibor, 1984; Saga & Sakai, 1984). These enzymes came from grazers such as *Halioltis*, *Aplysia*, and *Patella*, and from pathogenic bacteria and fungi that produce enzymes such as alginases, agarases, carrageenases and cellulases. However, in past experience, above-holdfast and sexual tissues of *Porphyra* (Polne-Fuller & Gibor, 1984) were partially resistant to the available enzymes: *Sargassum* subsurface and inner-cortical cells were impossible to dissociate, and the largest *Macrocystis* inner-cortical cells dissolved beyond recognition. Using the amoebal enzymes it was possible to dissociate the entire thallus of *Porphyra*, the subsurface and inner-cortical tissues of *Sargassum*, and the entire thallus of *Macrocystis*.

The amoebae displayed a clear preference for certain food sources such as *Macrocystis*, *Laminaria*, *Porphyra*, *Prionitis*, *Eucheuma*, and *Gracilaria* over *Sargassum*, *Egregia*, and *Gelidium*.

There was a lag period between feeding by the amoebae on a specific algal species and the acceptance of a different algal species. The length of the lag period depended on the digestibility of the seaweed, and its similarity to the previous seaweed on which it was fed. No lag phase of feeding occurred between the three species of *Eucheuma*, or between *Eucheuma*, *Gracilaria*, and *Prionitis*, but a lag or seven days occurred between *Gracilaria* and *Gelidium* although these are both agarophytes. A lag of one to two weeks occurred upon switching from *Macrocystis* to *Prionitis*, or from *Prionitis* to *Sargassum*. During the long delay in feeding on new food sources, the amoebae became colorless and stretched, an indication of starvation. In many cases the amoebae went through multiple fissions when transferred to a new food source, an indication of stress. In all cases, however, they eventually started feeding. After intensive visual observations it was concluded that the entire amoebal population recovered feeding activity, rather than a selected few individuals that could have been better able to utilize the new food source and might have taken over the population.

It was not determined whether new enzymes were induced or whether a relative quantitative shift of the different enzymes was taking place.

The decreased ability of the digestive enzymes to attack specific seaweeds was expressed in increased protoplast yields and shorter periods of incubation. The yields of *Sargassum* protoplasts increased over 500-fold when enzymes of amoebae that were grown on *Sargassum* were used rather than enzymes of amoebae grown on *Porphyra* (Fig. 1). A 38-fold increase in protoplast yield was evident from *Porphyra* when enzymes from amoebae fed on *Porphyra* were used rather than those fed on *Sargassum* (Fig. 1). No significant difference in protoplast yields was evident when either *Porphyra* or *Eucheuma* was treated with enzymes from amoebae that were fed on either one of these red seaweeds. This was true in spite of the different major carbohydrates in the cell walls, *Eucheuma* being rich in carrageenans whereas *Porphyra* has porphyrans, xylans, and mannans.

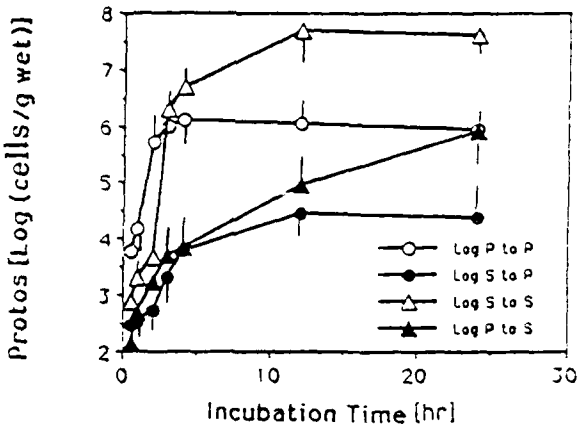

Fig. 1. Protoplast yields (Log) by digesting one gram of wet tissue in different enzymes. Clear circles = Enzymes from *Porphyra perforata*-fed-amoebae on *Porphyra perforata* (P-O-P). Dark circles = Enzymes from *Sargassum muticum*-fed-amoebae on *Porphyra perforata* tissues (S-●-P) Clear triangles = Enzymes from *Sargassum muticum*-fed-amoebae on *Sargassum muticum* tissues (S-△-S). Dark triangles = Enzymes from *Porphyra perforata*-fed-amoebae on *Sargassum muticum* tissues (P-▲-S).

Cuticles of live *Macrocystis* and *Sargassum* were digested with the rest of the tissues and seldom were found in the culture vessel. Cuticles of *Sargassum* were not separated from the tissues even after autoclaving. However, cuticles of autoclaved *Macrocystis* (Fig. 2) and those of live or autoclaved *Porphyra* (Fig. 3) were selectively left as a last food source (Table 1). In some of the amoebal cultures that were fed on brown and red seaweeds, an indigestible fraction was ejected from the food vacuoles as pellets of clear material ('feces'). Such particles were not membrane bound and did not contain detectable proteins. They melted at 40–65 °C depending on the food source, revealing their carbohydrate composition. Clear pellets were observed in cultures that were fed on *Macrocystis*, *Laminaria*, *Egregia*, *Gracilaria*, *Prionitis*, *Gelidium*, *Eucheuma* and *Porphyra* as well as on various agars, carrageenans, and alginates. The amoebae did not take up these pellets again. In cultures fed *Sargassum* and *Macrocystis* the amoebae released dark-brown pellets 5–10 μm in diameter. Further enzymatic and biochemical studies on the nature of these undigested materials are in progress.

Fig. 2. Cuticle of autoclaved *Macrocystis pyrifera* (clear arrow) being digested by amoebae (dark arrows) after the tissue has been consumed. Bar = 40 μm. *Fig. 3.* Cuticle of *Porphyra perforata* (clear arrow) being digested (dark arrows) after the rest of tissue has been consumed. Bar = 40 μm.

The mechanism by which the amoebae were 'attacking' the seaweeds and the gelled blocks of polysaccharides (2% in seawater) was observed. Whereas unicellular algae were phagocytosed individually, the large seaweeds had to be attacked enzymatically. In order for the amoebae to phagocytose a large seaweed or agar gels, they had to attach to the surface of the thallus or gel and create an external digestive pocket at the contact area; then they could dissolve a portion of the thallus or gel. Since enzymes could not be detected in the supernatant, it is assumed that they were either short-lived or possibly membrane-bound under natural feeding conditions. Enzymes were water soluble when the amoebae were ground in buffers or in sea water, and the activity remained in the centrifuged and filter-sterilized supernatant.

The feeding of these amoebae on a wide variety of seaweeds and the wide distribution of the genus in all warm temperate waters (isolated from seaweeds and sand samples from east and south

ible 1. Dissociation of seaweed tissues and cuticles by noebal enzymes.

:aweed ecies	Tissue dissociated	Cuticle digestion
rphyra	All tissues	Live & autoclaved cuticle digested last
rgassum	Surface and subcortical	Live & autoclaved cuticle digested with tissue
acrocystis	Surface, subcortical and medullary filaments	Live cuticle eaten with tissue; autoclaved cuticle digested last

ustralia, south Brazil, central and southern California, Hawaii, and Israel, unpublished) may oint at a potential pathogen to commercial eaweed farming of species such as *Macrocystis*, *aminaria*, *Eucheuma*, *Gracilaria*, and *Porphyra*. *Trichosphaerium* individuals have been isolated outinely from holes and blemishes on *Macrocystis* and *Laminaria* fronds in California coastal vaters. They were consistently isolated from thalli f *Gelidium*, *Prionitis* and *Gigartina*, mostly from oles and damaged parts of the tissues (not necesarily decaying), and from intact vegetative tissues nd receptacles of *Sargassum*. The succession of athogens in the tissues was not studied, but the bility of these amoebae to penetrate through uticles of the various seaweeds makes them a athogen at least by creating sites for further bacerial and fungal attacks.

Acknowledgements

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References

Cheney, D. P., E. Mar. & J. van der Meer, 1984. Protoplast production and culture in the agar-producing seaweed Gracilaria tikvahiae (Rhodophyta). J. Phycol. (Suppl.) 20: 21.

Fisher, D. & A. Gibor, 1987. Production of protoplasts from the brown alga, Sargassum muticum. Phycologia 26: 488–495.

Fujita, Y. & S. Migita, 1985. Isolation and culture of protoplasts from some seaweeds. Bull. Fac. Fish. Nagasaki Univ. 57: 39–45.

Kloareg, B., M. Polne-Fuller & A. Gibor, 1989. Mass production of viable protoplasts from Macrocystis pyrifera. Pl. Sci. 62: 105–112.

Liu, W. S., Y. Tang, X. W. Liu & T. C. Fang, 1984. Studies on the preparation and on the properties of sea snail enzymes. Proc. int. Seaweed Symp. 11: 319–320.

Polne-Fuller, M. & A. Gibor, 1984. Developmental studies of *Porphyra* species. I. Cell differentiation and protoplast regeneration in Porphyra perforata. J. Phycol. 20: 609–616.

Polne-Fuller, M., 1987a. A multinucleated marine amoeba which digest seaweeds. J. Protozool. 34: 159–165.

Polne-Fuller, M., 1987b. Cleaning of seaweed tissues (for protoplast research). In M. Indergaard, K. Ostgaard & M. Guiry (eds), Seaweed Protoplast and Tissue Culture; Proceedings workshop on biotechnology methods in seaweed cultivation. COST 48 'Aquatic Primary Biomass (marine macroalgae)', Trondheim, Norway: 31–32, 41–43.

Provasoli, L., 1968. Media and prospects for the cultivation of marine algae. In A. Watanabe & A. Hattori (eds), Proc. U.S.-Japan Conf.-Cultures and Collections of Algae: 63–75.

Saga, N. & Y. Sakai, 1984. Isolation of protoplasts from Laminaria and Porphyra. Bull. jap. Soc. sci. Fish. 50: 1085.